Universidade de Aveiro Departamento de Química
Ano 2016

JOÃO PEDRO LEÃO ARAÚJO DE ALMEIDA

VALIDAÇÃO DE UM NOVO OLIGOSSACÁRIDO DO LEITE HUMANO PARA USO EM FÓRMULA INFANTIL UTILIZANDO TECNOLOGIAS GASTROINTESTINAIS *IN VITRO*

Universidade de Aveiro Departamento de Química
Ano 2016

JOÃO PEDRO LEÃO ARAÚJO DE ALMEIDA

VALIDAÇÃO DE UM NOVO OLIGOSSACÁRIDO DO LEITE HUMANO PARA USO EM FÓRMULA INFANTIL UTILIZANDO TECNOLOGIAS GASTROINTESTINAIS *IN VITRO*

VALIDATION OF A NOVEL HUMAN MILK OLIGOSACCHARIDE FOR USE IN INFANT FORMULA USING GASTROINTESTINAL *IN VITRO* TECHNOLOGIES

Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biotecnologia, realizada sob a orientação científica da Doutora Sílvia Maria da Rocha Simões Carriço, Professora Auxiliar do Departamento de Química da Universidade de Aveiro, e do Doutor Pieter van den Abbeele e Beatriz Guimarães da empresa ProDigest, Gante, Bélgica.

o júri

a presidente

Prof.ª Dr.ª Ivonne Delgadillo Giraldo

professora associada com agregação no Departamento de Química da Universidade de Aveiro

Prof.ª Dr.ª Sílvia Maria da Rocha Simões Carriço (Orientadora)

professora auxiliar no Departamento de Química da Universidade de Aveiro

Prof.ª Dr.ª Marcela Alves Segundo (Arguente)

professora auxiliar no Departamento de Química da Faculdade de Farmácia da Universidade do Porto

palavras-chave

aparelho digestivo, cólon, microbiota intestinal, bebés; formulação; prebiótico, oligossacárido do leite humano, *in vitro*; s*imulator of the human intestinal microbial ecosystem* (SHIME), ácidos gordos de cadeia curta, amónio, lactato, DGGE, qPCR, *B. longum* subsp. i*nfanti*s.

resumo

O corpo humano abriga uma comunidade microbiana da qual, parte vive no aparelho digestivo. O cólon é a região com a comunidade bacteriana mais densa, denominada microbiota intestinal. O seu desenvolvimento em bebés pode ser influenciado por um número de fatores, tal como o ambiente intrauterino, tipo de parto e/ou o modo de alimentação. No que diz respeito ao modo de alimentação, o leite materno tem um papel importante na colonização intestinal de bebés através do fornecimento de uma variedade de oligossacáridos. Para bebés que não possam ser amamentados, uma formulação infantil é necessária como substituta e, portanto, deve satisfazer as necessidades nutritivas dos recém-nascidos.

A manipulação da microbiota intestinal, recorrendo à adição de probióticos e/ou prebióticos à dieta, tem-se tornado uma prática recorrente. Assim, este estudo teve como objetivo testar um novo oligossacárido do leite humano (NMO), via experiências gastrointestinais *in vitro*, a fim de proporcionar a melhor formulação possível para substituição do leite humano.

Inicialmente, uma experiência de pré-triagem foi realizada para obter informações sobre as potenciais diferenças inter-individuais entre bebés, em resposta à administração de NMO. No global, 7 dos 10 dadores responderam intensamente ao tratamento com NMO, o que resultou numa acidificação do meio. Um efeito bifidogénico foi também observado, com a degradação de NMO ocorrendo através de diferentes cenários de resposta. A escolha do dador 10 foi fundamentada tendo em conta a sua elevada taxa de fermentação e consequente produção de acetato, mas principalmente devido a um intenso efeito bifidogénico, mais especificamente, a um estímulo característico da *B. longum* subsp. i*nfantis*, após administração de NMO.

De seguida, um *baby M-SHIME*® com 5 unidades foi realizado utilizando amostras fecais de um único doador (bebé 10) como inóculo, tornando esta segunda parte do projeto ainda exploratória. Diferentes doses de um *"golden standard"* (GS) e NMO foram testados. O consumo base-ácido, as concentrações de ácidos gordos de cadeia curta (AGCCs), lactato e amónio e a composição da microbiota foram analisados. Durante o período de tratamento, 3.2 g/L de GS, NMO, ou combinações destes, foram adicionados aos reatores, resultando no aumento dos níveis de consumo de base-ácido e de AGCCs relacionados com a saúde. Um pico no lactato foi observado para as misturas e diminuições dos níveis de marcadores proteolíticos foram também observados. No que diz respeito a mudanças na composição de *Bifidobacterium*, GS provocou um estímulo de B. *longum*, enquanto NMO aumentou a abundância de *B. longum* subsp. i*nfantis*, com um efeito dose-resposta claro em ambas as situações.

No decorrer do tempo, a administração NMO causou também um aumento dos níveis de *Enterobacteriaceae* com relação dose-resposta. Das enterobactérias podem também fazer parte alguns agentes patogénicos e, sendo assim, a dosagem de NMO seria recomendada. A dose ótima pode, por conseguinte, ser a dose para a qual existe ainda uma forte estimulação de *B. longum* subsp. *infantis* e AGCCs relacionados com a saúde, embora não resultando numa grande expansão de enterobactérias, como, por exemplo, 75% / 25% ou 50% / 50% (GS / NMO).

keywords

human gastrointestinal tract, colon, gut microbiota, Infants, Formula, prebiotic, human milk oligosaccharide, *in vitro*, simulator of the human intestinal microbial ecosystem (SHIME), short chain fatty acids, ammonium, lactate, DGGE, qPCR, *B. longum* subsp. i*nfantis*.

abstract

The human body harbours a microbial community, part of which lives in the gastrointestinal tract. The colon is the region with the densest bacterial community, called gut microbiota. Its development in infants may be influenced by a number of factors, like intra-uterine environment, delivery mode and/or the feeding mode. Regarding the feeding mode, human breast milk plays an important role in early gut colonization of infants. It does so by providing a variety of human milk oligosaccharides. For infants who cannot be breastfed, infant formula is required as a substitute and so, must satisfy the nutritional requirements of infants.

Since modulation of gut microbiota resorting to the addition of probiotics and/or prebiotics to the diet is increasingly becoming a recurrent practice, in order to provide infants that do not receive breast-feeding with the best possible alternative formula feeding, this study aimed to test a new human milk oligosaccharide (NMO) via gastrointestinal *in vitro* experiments.

Firstly, a pre-screening experiment was performed to gain information on the potential inter-individual differences among babies in response to NMO administration. Overall, 7 out of the 10 donors responded strongly to the NMO treatment resulting in an acidification of the medium. A bifidogenic effect was also noted, with NMO degradation being found to occur via several different response scenarios. The choice of donor 10 was substantiated based on the strong overall fermentation and corresponding acetate production, but mainly due to a strong bifidogenic effect and thus, most interestingly, a specific stimulation of *B. longum* subsp. i*nfantis* upon NMO administration.

Afterwards, a 5 units' baby M-SHIME® experiment was performed using faecal sample from a single donor (donor 10) as the inoculum, making this second part of the research still exploratory. Different doses of a "golden standard" (GS) and NMO were tested. Base-acid consumption, short chain fatty acids (SCFAs), lactate, ammonium concentrations and microbiota composition were analysed. For the treatment period, 3.2 g/L of GS, NMO or combinations of thereof were supplemented to the vessels resulting in increased base-acid consumption and health-related SCFAs levels. A peak in lactate was observed for the mixtures and minor decreases were also observed on proteolytic markers. With respect to changes in *Bifidobacterium* composition, it followed that GS stimulated *B. longum*, while NMO increased the abundance of *B. longum* subsp. i*nfantis* with a clear dose-response effect in both situations.

Over time, NMO administration caused an increase of Enterobacteria levels in a dose-related way. Given the fact that Enterobacteria also contain opportunistic pathogens, dosing would be recommended. The optimal NMO dose might therefore be the dose at which there is still a strong stimulation of *B. longum* ssp. i*nfantis*, and health-related SCFAs, although not resulting in a major expansion of Enterobacteria, like for example 75% / 25% or 50% / 50% (GS / NMO).

INDEX

FIGURE INDEX

TABLE INDEX

I. INTRODUCTION

One of the most crucial shaping steps of human history was the discovery of bacteria, back in 1675 by a Dutch tradesman and scientist, named Antonievan Leeuwenhoek, the "Father of Microbiology". It still lasted until the 1800's, when for the first time, microorganisms were shown to cause disease. This was the trigger for a major scientific revolution leading to a better understanding, prevention and cure of microbial diseases resulting, for example, in improved hygiene, vaccinations and antibiotics (Dethlefsen et al., 2007).

However, most interactions between humans and microorganisms do not result in disease.

The interactions between host and non-pathogenic microorganisms are even essential to many aspects of normal mammalian physiology, therefore greatly influencing the being's health (Dethlefsen et al., 2007).

With this in mind, the human body harbours a microbial community which has been estimated to collectively consist of ten times more cells as compared to the number of human cells in a healthy adult (Qin et al., 2010). This microbial community, also called the human microbiota, includes archaea, fungi, viruses, protozoa and mainly, bacteria (Cénit et al., 2014). Due to the high volume, microorganisms are present in various body habitats such as in our skin surface. The gastrointestinal tract (GIT) is the most colonized part of the human body (Bianchi et al., 2014), with the colon harbouring a very dense and metabolically active microbial community called, the gut microbiota.

In contrast, the stomach is the region having the lowest bacterial density (10^1-10^3 cfu/mL), as schematized in Figure 1, since most bacteria are hindered by gastric secretions such as acid, bile or pancreatic juice (O'Hara and Shanahan, 2006). The gut microbiota rises up to an estimated 10^{11}–10^{12} bacteria per gram of colonic content, contributing to 60% of faecal mass (O'Hara and Shanahan, 2006).

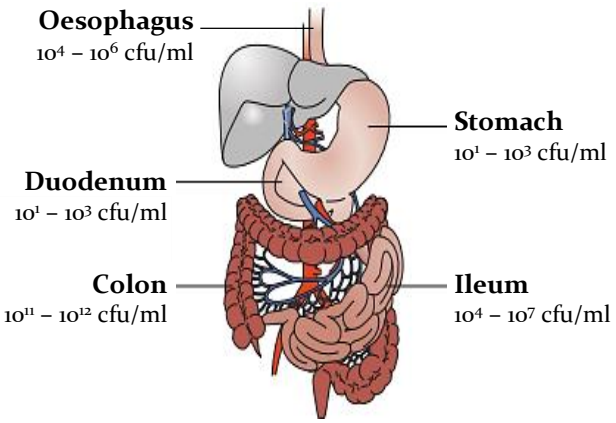


Figure 1 – Bacterial densities corresponding to different anatomic regions of the GIT (edited from O'Hara and Shanahan. 2006)

1. Gut Microbiota

1.1 Characterization

During a long term study, the composition of an individual's gut microbiota was shown to remain fairly stable throughout an adult's life (Martín et al., 2014). Aerobic and, predominately, anaerobic bacteria are present, with *Firmicutes, Bacteroidetes, Actinobacteria* (which includes the genus *Bifidobacterium*), *Proteobacteria* and *Verrucomicrobia* being the most dominating phyla (Cammarota et al., 2014).

Nevertheless, the bacterial population is continuously subjected to internal and external parameters that will affect the final structure and functions of the gut microbiota, making it unique. In order to fulfil specific niches, also other phyla colonize this microbiota including, *Chlamydiae, Cyanobacteria, Deferribacteres, Deinococcus, Thermus, Fusobacteria* or/and *Spirochaetes* (Dethlefsen et al., 2007). Altogether, about 1000 bacterial species reside in the human gut alongside with other microbial species (Cammarota et al., 2015).

The internal parameters that affect the gut's microbiota composition are residence time, pH, exposure to oxygen, nutrient availability, host secretions, and the presence and activity of immune cells and mucosal surfaces (Louis et al., 2014). Particularly interesting is the last parameter, as only few specific bacterial species are capable of colonizing mucus surfaces, resulting in a unique bacterial composition in the mucus layer versus the lumen. As the microbes in the mucus layer reside very close to the epithelial lining, they have a high potential to affect human health. Important mucosal microbes include butyrate-producing species (Van den Abbeele et al., 2013) and mucin-degrader bacterium, *Akkermansia mucinphila* (Sommer and Bäckhed, 2013). On the other hand, external parameters comprise environmental factors and the host's lifestyle, diet and age (O'Hara and Shanahan, 2006).

Further, in a comparative study of the gut microbiome of adults, the authors showed strong positive correlation between the microbial composition and the genetic relation of their hosts. The authors suggested that the host genotype also has a significant effect on the bacterial diversity, even prevailing over environmental factors (Erwin G. Zoetendal, 2001).

1.2 Function

The human gut microbiota plays a key role in numerous metabolic, physiological, nutritional and immunological processes, including fermentation of complex diet-derived host-indigestible substrates (mainly carbohydrates and proteins), synthesis of vitamins and maturation of immune cells for the normal development of host immune functions (Clarke et al., 2014; Marques et al., 2010). In addition, the indigenous gut microbiota can also prevent invasion of pathogens by competing for the colonization of the specific niches in the gut microbiome, thus preventing them from taking roots in the bowel (Cammarota et al., 2015).

One of the most important metabolites of gut microbes are short-chain fatty acids (SCFAs) (Pryde et al., 2002). The main SCFAs are acetic, propionic and butyric acids, which occur normally in 60:20:20 molar ratios. By absorbing and metabolizing SCFAs, the host is able to salvage energy (Clarke et al., 2014). Other possible effects of SCFAs on the human body is that they can affect epithelial cell transport and metabolism, epithelial cell growth and differentiation or hepatic control of lipid and carbohydrates (Clarke et al., 2014).

Butyrate is the SCFA which has been receiving most attention because it has important physiological functions in eukaryotic cells (e.g. histone acetylation) and, most importantly, because butyrate has anti-inflammatory and anti-carcinogenic effects on the human host (Flint et al., 2012; Mischke and Plosch, 2013). Butyrate is mainly produced by oxygen-sensitive anaerobes belonging to the *Clostridium* clusters IV and XIVa (also known as the *Clostridium leptum* and *Clostridium coccoides* groups, respectively). As schematized in Figure 2, two chemical pathways are responsible for producing butyrate: the butyryl-CoA/acetate-CoA transferase pathway, the most predominant, and the butyrate kinase pathway (Pryde et al., 2002; Vital et al., 2013).

Propionate is produced by *Bacteroides* species and members of the *Clostridium* cluster IX group, via the succinate and acrylate pathways, respectively (Fig. 2) (Van den Abbeele et al., 2011). Propionate is most known for having influence on lowering cholesterol and improving glucose homeostasis on the human host (Flint et al., 2012).

Regarding acetate, although it may be produced almost by any bacterium, its main producers are lactic acid bacteria, particularly Bifidobacteria and *Lactobacilli*, which are also able to produce lactate (lactate-producing bacteria). These bacteria are unable to produce either propionate or butyrate, however they are linked to their increase. By a process

called *cross-feeding*, acetate and lactate produced by lactic acid bacteria may be converted into butyrate and propionate, respectively, by lactate-utilizing microorganisms (Fig. 2).

Other SCFAs, including valerate, isobutyrate, formate or isovalerate can also be produced but in lower quantities, alongside with other end products such as ethanol, methanol and hydrogen sulphide (Louis et al., 2014) .

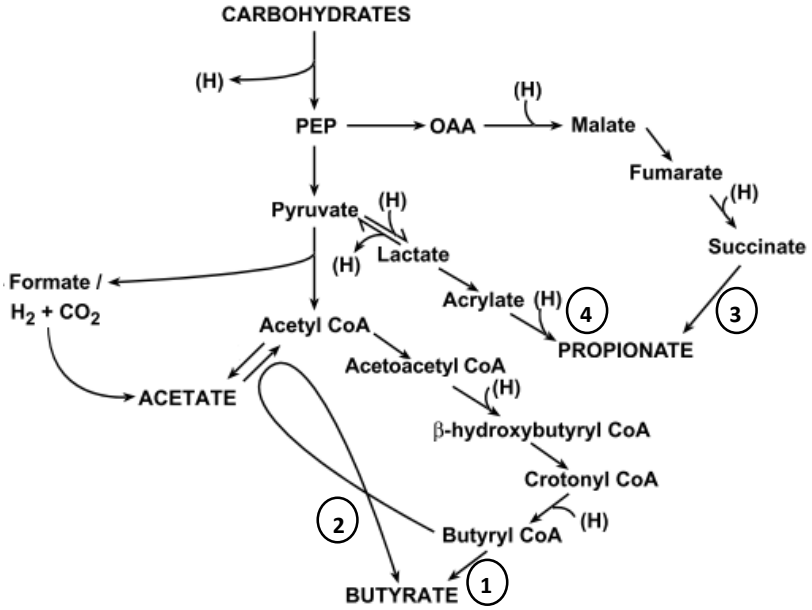


Figure 2 – Schematic representation of main pathways for carbohydrate fermentation and production of the three main SCFAs (acetate, propionate and butyrate) in the colon (edited from Pryde et al., 2002).
(1*) phosphotransbutyrylase/butyrate* kinase; (2) *Butyryl-CoA/acetate-CoA* transferase; (3) Acrylate pathway; (4) Succinate pathway.

Collectively, the gut microbiota exerts a metabolic activity that can be compared to a virtual organ within an organ (O'Hara and Shanahan, 2006). Therefore, perturbations in the gut microbial ecology (dysbiosis) may affect host resilience, gut permeability and alter the susceptibility to metabolic disorders resulting in adverse consequences for host health (Cani et al., 2008). Those consequences usually consist of pathological intestinal conditions such as chronic inflammatory diseases particularly, inflammatory bowel diseases, encompassing ulcerative colitis and Crohn's disease (Park et al., 2005). Such diseases can indeed be associated with specific alterations in the composition of the microbiota. As an example, in Crohn's disease patients, an outgrowth of the following bacterial taxa has been observed: species belonging to the *Bacteroides* genus, the *Enterobacteriales* order and to the *Enterobacteriaceae* family including pathogens belonging to the *Salmonella*, *Shigella* and

Escherichia genera (Cammarota et al., 2015). Further, in IBD patients, the changes in bacterial abundances have been linked to an alteration of metabolic functions of gut microbiota, such as a decrease in the metabolism of SCFAs and biosynthesis of amino acids, as well as an increase in auxotrophy, oxidative stress and secretion of toxins (Cammarota et al., 2015).

In addition to its local action, the action range of the gut microbiota goes even beyond the GIT. As demonstrated in Figure 3, clear links have been established between the composition of our intestinal microbiota, its functional endocrine interactions and the development of obesity, cardiovascular disorders and metabolic syndromes, as well as stress-related psychiatric disorders (Clarke et al., 2014). Especially the bidirectional communication system between the gut and the brain – the brain-gut axis – which is considered vital for maintaining homeostasis, is gaining a lot of interest by the scientific community (Zhou and Foster, 2015).

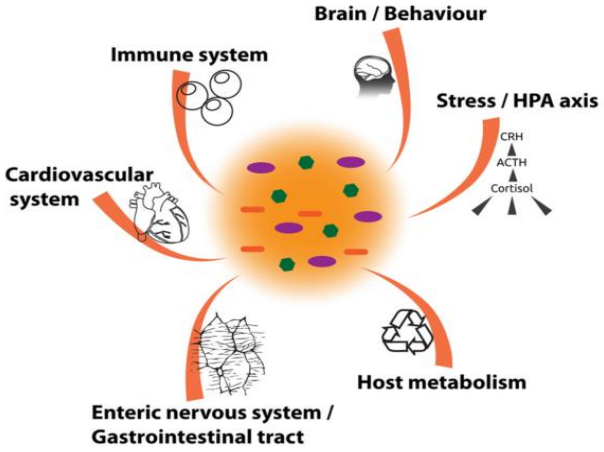


Figure 3 – Implications of endocrine output of the gut microbiota in health and disease (edited from Clarke et al., 2014).

In conclusion, the gut microbiota can no longer be considered a bystander in health and disease (Clarke et al., 2014).

1.3 Gut Microbiota Manipulation

The concept of modulating the human gut microbiota via the diet is not new. Three different approaches stood out in recent years: the addition of probiotics in foods, the selective stimulation of beneficial microorganisms indigenous to the gut with prebiotics, and a combination of both approaches (synbiotics) (Sivieri et al., 2014).

i. Probiotics

The term "probiotic" is a blend of Latin (*pro*=for, in favour of) and Greek (*bios*=life) (Ventura et al., 2015) and is officially defined as "live microorganisms which, when administered in adequate amounts, confer a health benefit on the host" (FAO/WHO, 2002).

Several key regulations were outlined by the FAO/WHO Working Group for the Evaluation of Probiotics in Food, specifically:

a. A probiotic must be alive when administered (although preparations of dead cells and cell components may also exert health-promoting physiological effects – *postbiotics*);
b. Probiotics must be delivered at an effective dose;
c. Probiotics must be taxonomically defined microbes or combination of microbes;
d. Probiotic must be safe for its intended use;
e. Probiotics must undergo controlled evaluation to document health benefits in the target host (Venema and do Carmo, 2015; Ventura et al., 2015).

Lactic acid bacteria have been the mostly investigated bacteria for use as potential probiotic (Bezirtzoglou and Stavropoulou, 2011). They can already be found in many products, such as dairy products. In regard to the mode of action of these or any other probiotics, it still is largely unclear what molecular effector determines whether one strain is active whereas another is not (Angelakis et al., 2013). However, possible mechanisms of action include that probiotics can prevent the colonization of pathogenic bacteria by decreasing intestinal pH, releasing antimicrobials (e.g. bacteriocins) or by competing for growth on substrates or for adhesion sites at the gut wall (Angelakis et al., 2013).

The prime example of lactic acid bacteria on probiotics is Bifidobacteria. Belonging to the genus *Bifidobacterium* (*Actinobateria* phylum) and first isolated in 1899 by Henri Tisser, they encompass 48 species, including four taxa (*longum*, *pseudolongum*, *animalis* and *thermacidophilum*) that are further divided into subspecies, all of which share more than 93% identity in their 16S rRNA sequences (Ventura et al., 2015).

Bifidobacterium is vastly distributed among living organisms that provide their offspring with parental care such as mammals, birds and social insects. This means that an important reason of their wide distribution may be due to direct transmission from parent to offspring (Turroni et al., 2011), which may also imply an important role of these bacteria in their hosts. In fact, in case of humans infants, colonization at an early stage by Bifidobacteria is known to be an important factor for health (Favier et al., 2003).

Several health benefits are attributed to the presence of Bifidobacteria in the human gut, including reduction of cholesterol, diarrhoea and constipation, lessening effects of

lactose intolerance, prevention of colon cancer, protection against infectious diseases, modulation of mucosal barrier function, amino acid and vitamin production, SCFAs production, and more (Hoover, 2014; Kondepudi et al., 2012; Leahy et al., 2005; Ventura et al., 2015).

Although the molecular basis of the mechanisms occurring between the intestinal host epithelium and Bifidobacteria is still unclear, all sequenced Bifidobacteria appear to encode an extracellular polysaccharides, cell envelope-associated structures, which may be crucial in bacterial adherence to host cells, as well as resistance to host secretions (Turroni et al., 2011). Bifidobacteria were also shown to present some fimbriae-like structures whose role has not yet been fully determined. Nonetheless, looking at other human GIT inhabitants which contain similar structures, one may expect that they also contribute to bacterial adherence and colonization on host cells surfaces (Turroni et al., 2011).

Recently, the EFSA (European Food Safety Authority) ruled out the possibility of using the word "probiotic" in any product. They claim that many products said to be probiotics contained only Bifidobacteria (and/or *Lactobacilli*) and had no substantial effect.

ii. Prebiotics

A different approach that has attracted interest is the use of prebiotics. The most recent definition for prebiotics given by the International Scientific Association of Probiotics and Prebiotics (ISAPP) is: "a selectively fermented ingredient that results in specific changes in the composition and/or activity of the gastrointestinal microbiota, thus conferring benefit(s) upon host health" (Śliżewska et al., 2012). The potential benefits resulting from the intake of prebiotics are schematized in Figure 4.

Prebiotics normally consist of carbohydrates (di, oligo and polysaccharides) that are non-digestible for the (human) host (Patel and Goyal, 2012). Prebiotics have to fulfil a few basic requirements. Firstly, they have to resist to gastric acids, host enzymes and gastrointestinal absorption. Further, they should be fermented by intestinal microbes and stimulate the growth and/or activity of specific intestinal bacteria (Roberfroid et al., 2010). Some examples of well-established prebiotics are summarized in Table 1.

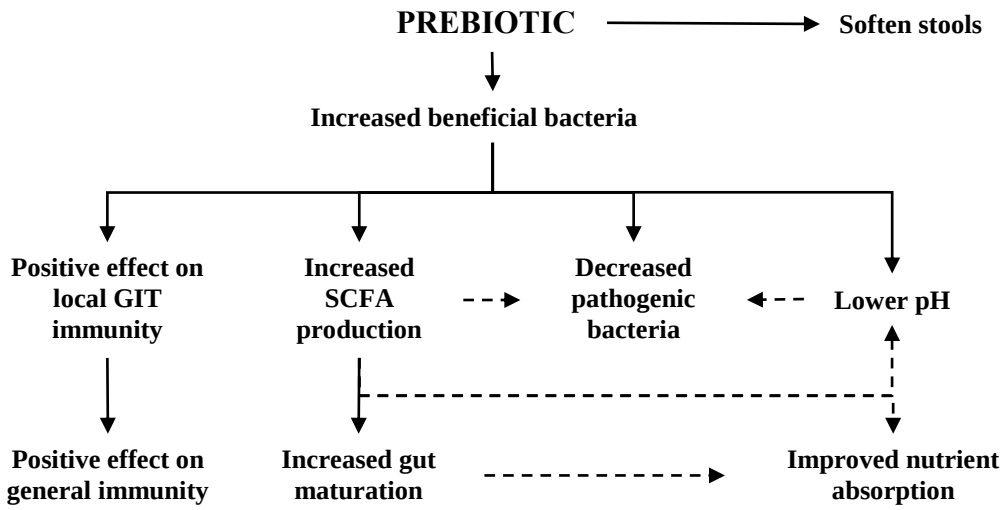


Figure 4 – Potential beneficial effects of prebiotics on infant health (edited from Donovan et al., 2009).

Prebiotic substrates offer several advantages over probiotic bacteria, including:

- Long shelf life;
- Heat and pH stable in a wide range of food products;
- Prebiotics enhance food taste and texture
- Prebiotics target specific strains that are already present in the intestinal tract of an individual, avoiding issues of probiotic that need to compete with intestinal bacteria that are well established in their niche (Śliżewska et al., 2012).

For these and other reasons, the prebiotic functional food market has grown into a multi-million Euro industry, with the prebiotic market accounting for an estimated €10.58 billion in 2012. By 2019, it is expected to grow to €15.03 billion (Transparency Market Research, 2014).

Nevertheless, until it can be officially released to the market, a prebiotic substrate must go through several stages, described in Figure 5. The goal of this process is to assess the safety of the product in the host and to confirm its health effects.

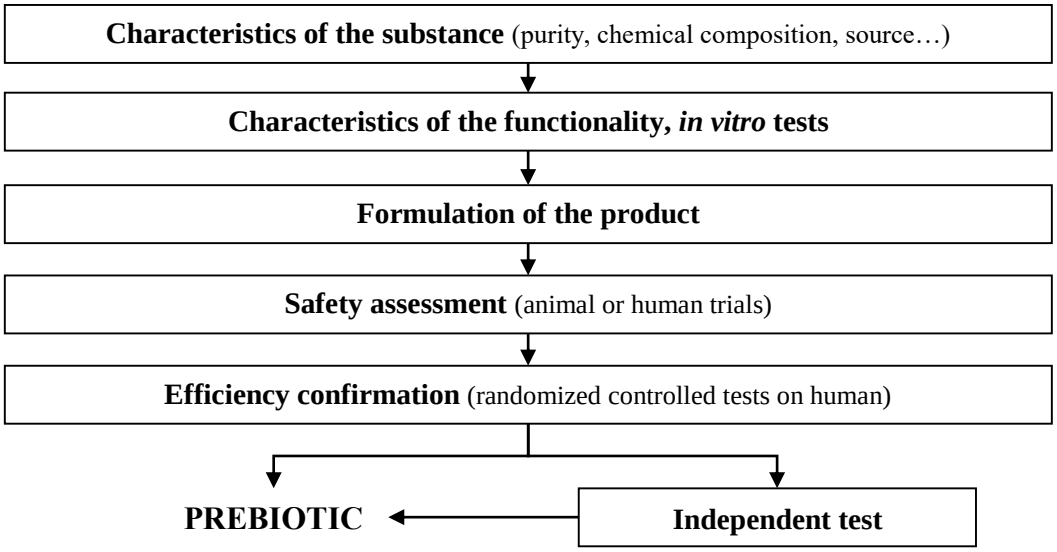


Figure 5 – Main guidelines to the assessment and proof of the action of prebiotics (edited from Śliżewska et al., 2012).

Table 1 - Most common and used prebiotics and their respective chemical structure and method of manufacture (edited from Śliżewska et al., 2012).

	CHEMICAL STRUCTURE	METHOD OF MANUFACTURE
Disaccharides		
Lactulose	β(1-4)-Fructans	Enzymatic treatment of lactose
Polysaccharides		
Inulin	β(2-1)-Fructans	Extraction from chicory root and *Agave tequilana*
Dextrin	Mixture of oligosaccharides containing glucose	Chemical modification of starch
Oligosaccharides		
Fructooligosaccharides (FOS)	β(2-1)-Fructans	Transfructosylation from sucrose or hydrolysis of chicory inulin
Galactooligosaccharides (GOS)	Galactose oligomers and some glucose/ lactose/ galactose units	Produced from lactose by β-galactosidase
Soya-oligosaccharides	Mixture of raffinose and stachyose	Extracted from soya bean whey
Xylooligosaccharides (XOS)	β(1–4)-Linked xylose	Enzymatic hydrolysis of xylan. Enzyme treatments of native lignocellulosic materials. Hydrolytic degradation of xylan
Isomaltooligosaccharides (IMO)	α(1–4)-glucose and branched α(1–6)- glucose	Microbial or enzymatic transgalactosylation of maltose. Enzymatic synthesis from sucrose

2. Early Life Microbiota

The assembly of a complex community of microorganisms in the gut is a nice example of how beneficial interaction is established between a developing microbial ecosystem and its host (Ventura et al., 2015). Formation of such a human-microorganisms symbiosis during early life is a complex and important biological process and should not be regarded as a succession of steps, but rather as a complex process influenced by microbial and host interactions and by external and internal factors (Fanaro et al., 2007).

Early colonizers of the gut typically include facultative anaerobes (or aerotolerants) belonging to Proteobacteria phyla, like *Enterobacteriaceae* and Streptococcus, whose number attains 10^9 CFU/g faeces (Bezirtzoglou, 1997; Fanaro et al., 2007; Kirmiz and Mills, 2016). From then on, the infant's gut microbiome will undergo rapid maturation over the first year of age, moving toward an adult-like microbiota within the first 3 years after birth (Matamoros et al., 2013).

The gradual consumption of oxygen in the intestine by aerobic microorganisms decreases the oxidation-reduction potential, thus providing optimal conditions for the settlement of a more diversified microbiota (Lopetuso et al., 2013). After the colonisation of facultative anaerobes, obligate anaerobic bacteria expand, changing the general structure of the gut microbiota. This new complex microbiota dominated by obligate anaerobes, mainly Bifidobacteria, promotes the establishment of butyrate producers by cross-feeding and provides a barrier against the proliferation of new non-beneficial bacterial strains, a phenomenon termed "*colonization resistance*" (Adlerberth and Wold, 2009).

It should be noted though, that such shifts in microbial composition may represent an outgrowth of specific groups of bacteria that were already present, albeit at low level. That is, for instance, the case for Bifidobacteria, whose abundance increases from 3.5-10% in the infant microbiota to about 50–70% during the first month age after birth (Nylund et al., 2014). At 3 months age, Bifidobacteria compose even 90% of the microbiota (Nylund et al., 2014). After the weaning and introduction of solid foods, Bifidobacteria gradually decrease in abundance reaching 60% at 4 months of age, 25% at 6 months and 10% at 2 years. At that time, microbial signatures start stabilizing and resembling the 'adult state' (Sommer and Bäckhed, 2013)

Even though the stabilization to an adult-like composition is reported to take place on this short notice, a few studies still suggest that it lasts throughout the entire childhood and adolescence, since major physiological changes are taking place in the human body. In a recent study, Hollister et al. (2015) compared the gut microbiotas of healthy adults versus healthy pre-adolescent children (age 7-12 years). They showed that both composition and functional potential were significantly different in both age groups, although they both harboured similar numbers of taxa and functional genes. Children were enriched in *Bifidobacterium* spp., *Faecalibacterium* spp. and members of the *Lachnospiraceae*, while adults harboured greater abundances of *Bacteroides* spp. These findings suggest that gut microbial community may undergo a more prolonged development than previously suspected.

As mentioned before, a number of external factors may influence the process of microbial colonization in infants. These factors, listed in Table 2, may affect the infant's health, reason for they should always be taken into account.

Table 2 – Summary of the factors affecting gut microbiota colonization in infants (edited from Munyaka et al., 2014).

Factors affecting colonization of gut microbiota before birth	**Factors affecting colonization of gut microbiota at birth**	**Factors affecting colonization of gut microbiota after birth**
-Intra-uterine environment -Maternal exposures or practices such as stress, antibiotic use, smoking -Length of gestation period (term vs preterm)	-Mode of delivery (caesarean section vs vaginal delivery) -The environment at the time of delivery -Contact with the mother or health care staff	-Breastfeeding vs formula feeding -Weaning or food supplementation - Antibiotic exposure - Home or family setting (rural vs urban) - Home structure (contact with family members)

2.1 Intra-uterine environment

Vertical transmission of maternal microbiota is the most important contribution to the genesis of an individual´s microbiota (Gritz and Bhandari, 2015). However, until now, the general idea was that the human foetus was microbiologically sterile before birth and that the passage of the infant through the birth canal was the only process responsible for colonization of new-borns. Likewise, the presence of microbes in the amniotic fluid and placenta was thought to be only associated with preterm deliveries due to maternal intrauterine infections (Nylund et al., 2014). Nonetheless, recent studies have shown that DNA of non-pathogenic bacteria can be detected in placenta and amniotic fluid samples even in normal conditions (Nylund et al., 2014). Hence, because foetuses begin to swallow large amounts of amniotic fluid as they become more neurologically mature, we can, therefore, affirm that the ingestion of amniotic fluid during pregnancy already exposes the foetus to bacteria (Gritz and Bhandari, 2015). In 2001, Aagaard and colleagues confirmed this idea after they characterized a placental microbiome profile, finding that this microbial community shared some similarities with the human oral microbiota (Aagaard et al., 2014). Both were composed of non-pathogenic commensal microbiota from the *Firmicutes*, *Tenericutes*, *Proteobacteria*, *Bacteroidetes*, and *Fusobacteria* phyla.

Several reports have also described the microbiota composition from the very first faecal specimen produced by infants after birth, the meconium, which consists mainly of amniotic fluid but includes also mucus, intestinal epithelial cells and concentrate of metabolites such as bile acids and pancreatic secretions (Nylund et al., 2014). Accordingly, they concluded that bacteria belonging to the major bacterial phyla in the intestine are already detectable which, once again, reinforces the idea that the colonization process of human foetus begins well before delivery.

Despite aforementioned findings, this idea is still not 100% accepted because the presence of bacteria in the amniotic fluid could also reflect an undetected infection. The presence of bacterial species in the meconium, such as *Escherichia coli*, *E. faecium*, and *Staphylococcus epidermidis*, could result from the translocation of the mother's gut bacteria via the bloodstream (Munyaka et al., 2014).

2.2 Delivery mode

Either with intrauterine influence or not, the mode of delivery is another factor that can affect the establishment of the infant gut microbiota, as it has been shown that babies born by caesarean section (C-section) develop a microbiota different from babies vaginally delivered, alongside with inefficient short-term immune responses and a greater long-term risk of developing immune diseases (Clarke et al., 2014). This proves that direct transmission of the vaginal microbiota provides the baby with an initial set of bacteria, occupying niches and reducing the colonization by pathogens as site-specific communities develop.

Dominguez-Bello et al. (2010) compared the initial microbiota of new-borns immediately after birth. As expected, they confirmed that the dominant taxa found in infant communities were reflective of delivery mode. *Lactobacillus*, *Prevotella*, *Atopobium* or *Sneathia spp.* were abundant in aggregate samples from vaginally delivered babies, and typical skin taxa, including *Staphylococcus spp.*, appeared in samples from C-section infants.

Most vaginal and skin bacteria do not seem to take hold in the infant gut but, their initial presence may differentially affect the colonization capacities of other bacteria (Francino, 2014). Indeed, in surveys performed at 3 days and 1 month of age, *Penders et al.* found that infants born by C-section were much less likely to be colonized by *Bifidobacterium* and *Bacteroides* at both times. These findings may, in part, explain why susceptibility to certain pathogens is often higher in C-section than in vaginally delivered infants.

2.3 Feeding mode

Another strong, and possibly the major one, factor influencing the development of the infant intestinal microbiota is the mode of feeding, with studies suggesting differences in microbial colonization of breast-fed versus formula-fed infants.

Breast-feeding is associated with numerous beneficial effects on the new-born (Kirmiz and Mills, 2016). Studies have linked breast-feeding with a reduction in the risk of asthma, obesity, type 1 and 2 diabetes and necrotizing enterocolitis, among other health benefits (Kirmiz and Mills, 2016). One of the explanations given for these wide range of benefits is the bacterial content present in breast milk. Collado et al. (2009) confirmed this while characterizing the breast milk microbiota of 50 healthy women. *Staphylococcus*, *Streptococcus*, *Bifidobacterium* and *Lactobacillus* were the most predominant groups.

Furthermore, genotyping of bacterial isolates from the breast milk of mothers and faecal samples of their infants revealed the presence of identical strains, suggesting once again an important role of breast milk as a source of early gut colonization in infants.

Human breast milk is also an important source of human milk oligosaccharides (HMOs), the third most abundant component of human breast milk after lactose and lipids (Underwood et al., 2015). They are believed to act as selective 'natural' prebiotics therefore promoting the colonization of the breast-fed infant gut with beneficial microbes as opposed to children fed with formula (Munyaka et al., 2014). Bifidobacteria, is the major bacterial group able to utilize such milk oligosaccharides (Nylund et al., 2014). Ward et al. was the first to prove this by showing that Bifidobacteria, in that case *Bifidobacterium infantis* ATCC 15697, could grow to high cell densities *in vitro* on HMOs as a sole carbon source. In another study, De Leoz et al. (2015) examined infant faecal HMOs in relation to faecal bacterial population in two healthy infants over the first few weeks of life. By week 13 they observed that there was a decrease in faecal HMOs, whereas *Bifidobacterium* spp. and also *Bacteroides* spp. had increased.

When comparing the faecal microbiota of breast-fed and formula-fed infants (n=12), Bezirtzoglou et al. (2011) found that they differ significantly in both composition and diversity using fluorescence in situ hybridization. It was concluded that breast-fed infants harbour a faecal microbiota dominated by Bifidobacteria (72.25%), with abundances being two times higher as compared to formula-fed infants. Finally, the microbiota of breast-fed infants might be more complex than previously thought. This relates to the finding of high levels of *Ruminococci* in breast-fed infants 1 month old (n=39). The authors suggested that *Ruminococci* might have a similar major role as Bifidobacteria, in breast-fed babies (Coppa et al., 2011).

Furthermore, healthy formula-fed infants are colonized by a more diverse population, including also bifidobacterial species seen in adults such as *B. adolescentis*. In contrast, healthy breast-fed infants are colonized by a small number of subspecies including *B. infantis*, *B. longum*, and *B. breve* and to a lesser extent *B. bifidum* and *B. pseudocatenulatum*. This is in accordance with the enhanced capacity of HMO consuming *B. infantis*, which is considered the archetypical HMO-utilizing bacterium (D. a Sela et al., 2008).

As already mentioned, the introduction of solid food causes a new shift in the gut microbiota of children evening out the differences between formula and breast-fed babies.

Yet, the gut colonization depends on the nature of food received, which can be stratified by cultural influences or geographical location. De Filippo et al. (2010) investigated and compared human intestinal microbiota from two groups of children characterized by a modern western European diet (Italy) and a rural African diet (Burkina Faso), respectively.

The graphs presented in Figure 6 show meaningful differences between the two groups, indicating a significant enrichment in *Bacteroidetes* and depletion in *Firmicutes* in rural African children. Moreover, a unique abundance of bacteria from the genus *Prevotella* and *Xylanibacter*, known to contain a set of bacterial genes for cellulose and xylan degradation was specifically found in African children, while completely lacking in European children. The authors suggested that gut microbiota most probably coevolved with the polysaccharide-rich diet of Burkina Faso individuals, allowing them to maximize energy intake from fibres.

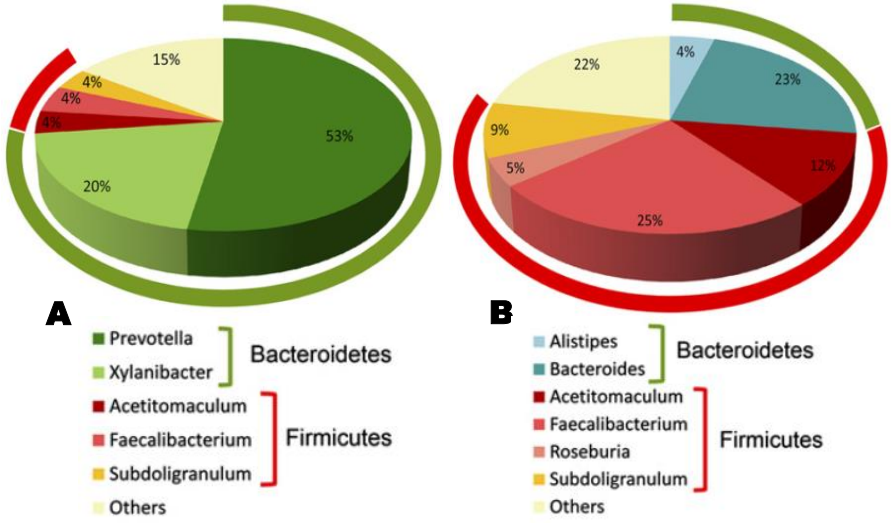


Figure 6 – Pie charts of median values of 16S rRNA gene surveys showing bacterial genera present in faecal samples of children from A) Burkina Faso and B) Italy (edited from De Filippo et al., 2010)

3. Supplemented Infant Formula

For infants who cannot be breastfed, infant formula is required as a substitute for mother's milk and, for that reason, infant formula must satisfy the nutritional requirements of infants (Koletzko, 2015). However, in contrast to human milk, cow's milk (usually used in infant formula) contains only trace amounts of oligosaccharides and so, to fulfil this gap, oligosaccharides must be provided exogenously, although it is not possible to add oligosaccharides structurally identical to HMOs (Donovan et al., 2009). Therefore, researchers have been evaluating the feasibility of adding food-grade prebiotic ingredients to mimic the functional properties of HMOs in infants' gut microbiota.

Regarding safety questions of this specific issue, the accumulated data concluded that the use of currently used formula with added prebiotics in healthy infants does not raise safety concerns (Braegger et al., 2011).

Several food-grade oligosaccharides have been evaluated and are currently in use as prebiotics in infant formula, mainly GOS and FOS, though inulin and lactulose have already been studied. Combinations of these products have also been evaluated. Prebiotic oligosaccharides have only been added to infant formulas in Europe within the first decade of the 21st century, although 90% of infant formulas in Japan already contain prebiotics (Ziegler et al., 2007).

Many studies have evaluated the effectiveness of individual prebiotics on gut microbial composition. In one study, Ben et al. (2008) decided to investigate the effect of only 2.4 g of GOS per 1L of formula on intestinal microflora colonization and fermentation in formula-fed term infants compared with breast-fed and control formula-fed counterparts. Infants were followed up for 3 months and, when not breastfed, some were randomly assigned to test formula group and control formula group. At the end of the feeding period the results, presented in Table 3, show that even with low levels of GOS, the number of intestinal Bifidobacteria (and also *Lactobacilli*) was significantly increased both in GOS-supplemented formula-fed infants and in breast-fed infants, compared with those fed with the control formula.

Table 3 – Levels of intestinal bacteria at the end of a 3 months feeding period as measured in fresh faeces (edited from Ben et al., 2008)

	GOS formula	Human milk	Control formula
Bifidobacterium	9.01 ± 1.18	9.25 ± 0.93	8.16 ± 0.99
Lactobacilli	5.91 ± 1.61	5.45 ± 2.16	4.27 ± 2.02

(Note: Data are presented as mean ± SD Log10 CFU/g wet faeces. Control formula does not contain added GOS)

A more recent work had similar results. The study consisted on randomly assigning infants to receive an infant formula until 6 months of age and then the follow-on formula until 12 months of age either with GOS supplementation (GOS group) or without (control group). As expected, Bifidobacteria increased in infants in the GOS group compared with the control group, although, this time no general differences were observed for *Lactobacilli* (Sierra et al., 2015).

In a different study, the authors decided to analyse the effect caused when feeding infants with a formula containing a combination of GOS and FOS in a 9:1 proportion (4 g/L) (Holscher et al., 2012). Formula-fed infants were randomized to consume a partially hydrolysed formula with or without the prebiotic and compared to a breast-fed infants group. In the end, stool of infants consuming the prebiotic clearly had the best results. Figure 7 shows us that the prebiotic consuming group (PRE) had a higher absolute number and proportion of Bifidobacteria than non-consuming prebiotic groups (CON and BF). Moreover, the amount of Bifidobacteria on faecal material did not differ significantly between breast-fed infants and prebiotic consuming group.

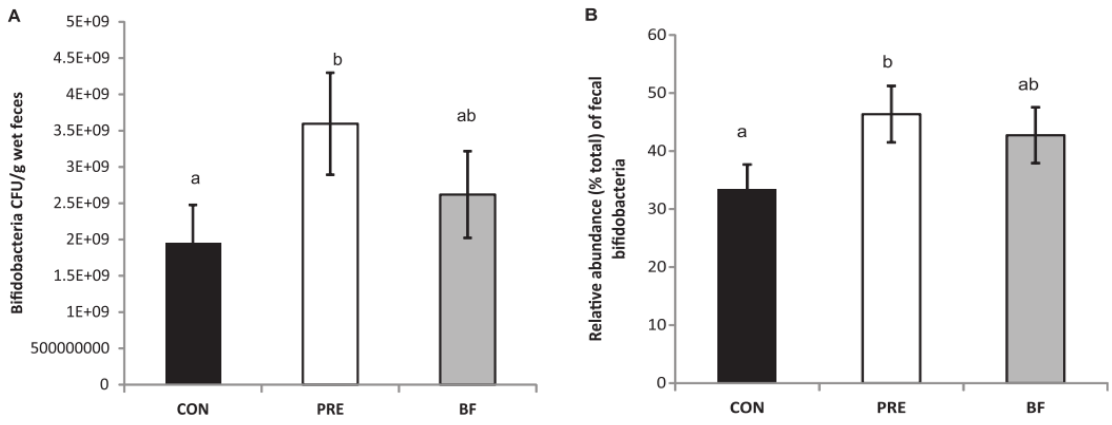


Figure 7 – A) Absolute abundance (CFU/g) and **B)** Relative abundance (percentage of total) of faecal Bifidobacteria in breast-fed infants and infants consuming prebiotic and control formulas (edited from Holscher et al., 2012). *BF:*breastfed; *CON:*control infant formula group; *PRE:*prebiotic infant formula group.

In a study aiming to test inulin as prebiotic in an infant formula, formula-fed infants (n=56) were enrolled to receive either the prebiotic or placebo (Kapiki et al., 2007). Faecal samples were taken at inclusion day and seven days later. Once again, faecal bacterial counts showed that the microbiota of supplemented infants were more heavily colonized with Bifidobacteria and had fewer potentially pathogenic bacteria (*E. coli* and *Enterococcus*) compared to infants fed with non-supplemented control formula.

Moro et al. investigated the effect on bacterial growth caused by the supplementation of different dosages of prebiotic, specifically, GOS:FOS (9:1). For that, infants were randomized to receive control formula or formula containing either 4 g/L or 8 g/L of GOS:FOS. At the end of a 28-day treatment period, the number of Bifidobacteria in the stools was significantly higher in both groups fed the supplemented formulas than in the stools of the placebo group. This effect was also dose-dependent and significantly different between the supplemented groups.

Summing up, HMOs have a crucial role in the development of formula-fed infants' gut microbiota, leading to increased levels of beneficial bacteria, particularly Bifidobacteria. It should be noted, however, that differences in one or more studies' results may show up but the complex gastrointestinal microbiota must always be taken into account. Also, it is important to keep in mind that genetic differences among infants are likely to also influence initial colonization and response to prebiotics and that all the results are based on bacterial counts on faecal samples as we know, for a fact, that these don't represent 100% accurately what goes on in the gut.

4. *In Vitro* Models

As mentioned, the majority of studies of the gut microbiota are based on bacterial counts on faecal samples. Because of this, the results are not able to provide us insights into dynamic microbial processes and functionality or digestion at their locations in the gut (Verhoeckx et al., 2015). For this purpose, *in vitro* fermentative models were developed as simulators of the ecology of the human colon offering possibilities of studying the interactions of substrates or strains with the native colon microbiota´s metabolic activity (production of SCFA, gases, enzymes, bacteriocins, etc.) and composition, without any ethical constraint (Alander et al., 1999).

In vitro models comprise two different types of models: batch and continuous models, both aiming microbial modulation and metabolism studies, resorting to controlled environmental conditions (Verhoeckx et al., 2015).

4.1 Batch Models

Batch models usually consist of closed-bottles incubations in anaerobic conditions using dense faecal microbiota. This is a simple way of characterizing the effect of substrates on microorganisms' physiology and biodiversity and, for that reason, is commonly used as a first assessment of the types of microbial metabolites formed (Verhoeckx et al., 2015). Batch models are often applied when we want to check for inter-individual variability in the response to a particular substrate or for comparison after exposure to different compounds or doses of the same compound (Verhoeckx et al., 2015). This model's implementation on longer simulations is not recommended because batch cultures are static and conditions may not be physiologically relevant at late time points (Aura et al., 2006).

4.2 Dynamic Models

The inclusion of host digestive functions *in vitro*, coupled with multistage continuous fermentation modelling, represents the most advanced attempt, thus far, at simulating interdependent physiological functions within the human gut (Payne et al., 2012). In this way, is now possible to study gut microbiota's long-term responses to substrates regarding metabolic activity and microbial community's variation.

Today, the main examples of continuous fermentation models are the TIM-2, a proximal colon simulator, and the SHIME (Verhoeckx et al., 2015).

i. SHIME

The SHIME® (Simulator of the Human Intestinal Microbial Ecosystem), schematized in Figure 8, is a five- stage sequential batch mode reactor system, operated at 37ºC, simulating the different parts of the gastrointestinal tract, based on the model developed by Molly and colleagues, in 1994 (Bianchi et al., 2014). The first two reactors simulate the stomach and small intestine, respectively, both being connected to a three-stage large intestinal model simulating the lower (ascending, transverse and descending colons) digestive tract (Payne et al., 2012).

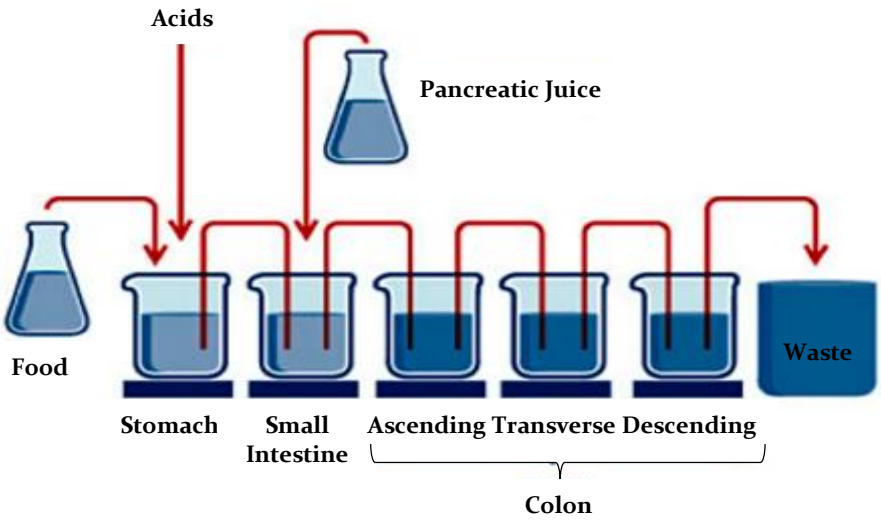


Figure 8 – Basic schematization of the Simulator of Human Intestinal Microbial Ecosystem (SHIME) (edited from *prodigest.eu*).

All reactors consist of double-jacketed glass vessels that are connected through peristaltic pumps. A defined nutritional medium (feed), composed of complex carbohydrate and protein sources with addition of mucins and a mineral and vitamin mix, is added three times a day to the stomach, in the same way as pancreatic and bile liquid (pancreatic juice) are added to the small intestine (Wiele et al., 2015). Upon digestion, the slurry is pumped into the ascending colon vessel where colon digestion is initiated, while all vessels are continuously mixed with magnetic stir bars, pH controlled and kept anaerobic by daily flushing the headspace of the respective compartments with N_2 gas (Wiele et al., 2015).

Because of the inaccessibility of the human colon region to take a representative microbial inoculum, faecal microbiota is chosen as inoculum to the colon compartments of the SHIME reactor. However, the idea of the SHIME system is to allow a suitable adaptation period for the faecal microbiome to adapt to the conditions that prevail in the respective colon compartments, so it can be the most accurate possible. Faecal samples are usually provided by a single individual due the enormous functional redundancy of the gut microbiome. This redundancy makes a pooled microbiome take on a fermentation profile very similar to the microbial fermentation profile of a single individual.

A typical SHIME experiment consists of four stages: stabilization, control, treatment and wash-out. During the stabilization period, a strict control of environmental conditions (e.g. nutrients, residence time, pH, temperature) is applied, in order to the human faecal inoculum evolve to a stable *in vitro* microbiota that is representative for the different colon regions of interest. A stabilization of the microbiota in terms of composition and metabolic activity is assured during the control period. Then, a specific alteration is applied to the system (treatment). From that moment, changes on the results observed are due to this new condition to which the microbiota is subjected. The wash-out period serves to determine how long the changes induced can still be measured after the treatment. In the end of a project, for a SHIME evaluation, general fermentation activity and metabolic potency are analysed, together with microbial composition of each colon vessel. Figure 9 displays a hypothetical example of a specific feature of bacterial metabolic activity changing throughout the different periods.

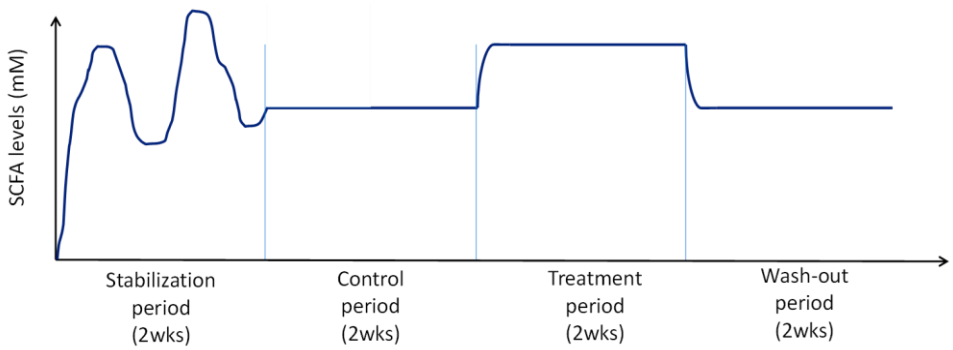


Figure 9 - Hypothetical example of microbial activity throughout a SHIME experiment. Upon inoculation of the fecal sample, the community is stabilizing resulting in variable metabolite levels. After 2 weeks, a steady-state is reached. At this point, the gut microbiome will only change when a treatment is initiated, allowing to establish causal relationships.

In order to investigate two different compounds at the same time, a TWINSHIME setup was developed by operating two systems in parallel at the same time (Van den Abbeele et al., 2010). Identical environmental conditions for both systems are obtained by identical pH, liquid transfer and temperature control.

Van den Abbeele et al. (2013) optimized the SHIME for mimicking mucosal microbial colonization by incorporation of mucin-covered microcosms. Using this new mucosal M-SHIME version, the author concluded that colonization of the mucosal environment was characterized by a higher abundance of butyrate producing *Clostridium* clusters IV and XIVa. Also, he demonstrated that the M-SHIME was able to maintain the unique features of an individual's microbiome in terms of its mucosal composition.

By altering some of the conditions or the structure, the SHIME can also be refined according to the subject being studied. De Boever et al. (2001) developed the Baby SHIME, destined to the study of gastrointestinal microbiota of infants.

The SHIME model has been validated across the years. The first validation coming from Molly et al. (1994), in which the authors compared fermentation profiles from a SHIME experiment with fermentation profiles from incubations with faecal microbiota from human volunteers. Since then, a couple of new validation papers have also been published, in 2006 and 2013, respectively (Possemiers et al., 2006; Van den Abbeele et al., 2013).

In Table 4 are presented advantages and disadvantages of the SHIME system.

Table 4 - Advantages and disadvantages to the SHIME system (edited from Wiele et al., 2015).

Advantages	– Integrates the entire gastrointestinal tract;
	– Microbiome inoculation from different target groups: adult vs. infant, healthy vs. diseased and animals;
	– Colon-region specific research;
	– Maintains microbiome stability over a long timeframe: possibility to monitor microbiome adaptation;
	– Mechanistic research by multi-parametric control;
	– Differentiation between mucosal and luminal microbiome in M-SHIME;
	– Parallel control and treatment in TWIN-SHIME setups;
	– Inter individual variability can be studied in a SHIME setup as unique features of an individual's microbiome are preserved. Limiting microbiome simulation to one colon region, eight different subjects can be simultaneously assessed.
Disadvantages	– Conventional SHIME setup lacks dialysis. Incorporation of dialysis modules is possible after small intestine digestion and colon digestion;
	– Lack of peristalsis;
	– Absence of host cells in conventional SHIME. Solved by coupling to HMI module with epithelial or immune cells.

II. AIM

In order to provide infants that do not receive breast-feeding with the best possible alternative formula feeding, this study aims to test a new human milk oligosaccharide (NMO) via gastrointestinal *in vitro* experiments. Firstly, resorting to short term experiments using faecal samples of 10 different babies, the intention was to understand how many donors responded positively to the treatment. This response was evaluated based on multiple endpoints specifically the pH, the SCFAs concentration and the *Bifidobacterium* spp. concentration and composition.

Subsequently, one donor inoculum was selected for a long-term baby M-SHIME experiment, making this second part of the project only exploratory. During this experiment, the NMO was compared to a 'golden standard' (GS) oligosaccharide. The evaluation of the results was based on the same endpoints as the previous experiment plus the lactate and ammonium concentrations, the base-acid consumption and the *Bacteroidetes*, *Firmicutes*, *Lactobacilli* and *Enterobacteriaceae* concentrations and compositions.

For confidential reasons both tested products cannot be identified.

III. MATERIALS & METHODS

1. Fed Batch

100 mL of buffer were added to 7.5 g of fresh faecal inoculum from each donor, followed by a vigorous mixing in the stomacher, for 2 min, and centrifugation for 2 min at 500 G. The supernatant was collected.

From the inoculum suspension, 40 mL were added to 10 mL of anaerobic cryoprotectant, well mixed in the vortex and distributed over 5 tubes. The tubes were then placed in liquid nitrogen at -80ºC, for preservation of the inocula.

Previous to the start of the batch, 30 penicillin bottles were filled with 63 mL of colon medium and incubated at 37ºC and 90 rpm, for 15 min. To each bottle, 7 mL of faecal inoculum were added. To 20 bottles (2 bottles per donor), 350 mg of the tested product were added - treatment bottles – for a final concentration of 5 g/L (Table 18, Appendix I). The bottles were flushed to create anaerobic conditions. Then, at different time points (0h, 24h and 48h), 4 mL samples were collected from each bottle for pH quantification, DNA extraction and SCFA analysis. Other 8 mL samples were also collected as backup.

2. Baby M-SHIME

The reactor setup was adapted from the SHIME® (ProDigest and Ghent University, Ghent, Belgium), in order to represent the GIT of the human baby, as described by De Boever et al. (2001). For these experiment, a baby M-SHIME setup was started operating seven proximal colons (PCs) at the same time (5 + 2 as backup that were later excluded), in order to obtain identical environmental conditions. Two extra Stomach/Small Intestine (S/SI) reactors were added. The whole setup, respectively showed and schematized in Figures 10 and 11, was autoclaved.

Before starting, the seven colon vessels were filled with 140 mL of autoclaved L-SHIME 6A nutritional medium (or feed) (Table 19, Appendix II). Taking into account the absence of absorption in small intestine, the feed included “pre-digested and pre-absorbed” milk compounds in the medium rather than raw infant formula. Once the pH of the vessels reached values in the range of 5.4 and 5.6, 10 mL of the bacterial inoculum (donor 10) was introduced. Two times per day, feed and pancreatic juice (PJ) (12.5 g/L NaHCO3 (Difco), 4 g/L bile salts (Difco) and 0.9 g/L pancreatin (Sigma Aldrich®)) were added to the S/SI

compartments, and then transferred to each vessel. In each cycle, an equal amount was taken out of the vessel as waste. In this way, the final residual time was 12 hours. All the vessels were kept anaerobic by flushing them with N_2, were continuously stirred (300 rpm) and kept at constant temperature (37ºC) and pH (5.6-5.8). Over a period of twenty days, every two days, content from each vessel was collected always at the same time and stored at -20ºC for metabolic and microbial analysis.

Just as a M-SHIME, additionally, each PC included a mucus compartment as the inclusion of a mucus layer increases the relevance of the simulation and proved to deliver additional results. The presence of the mucus appears to be an important factor for Bifidobacteria, including *Bifidobacterium longum* subsp. i*nfantis* (Kavanaugh et al., 2013; Muñoz et al., 2011; D. A. Sela et al., 2008), potentially providing this strain a selective advantage in colonizing the gastrointestinal tract.

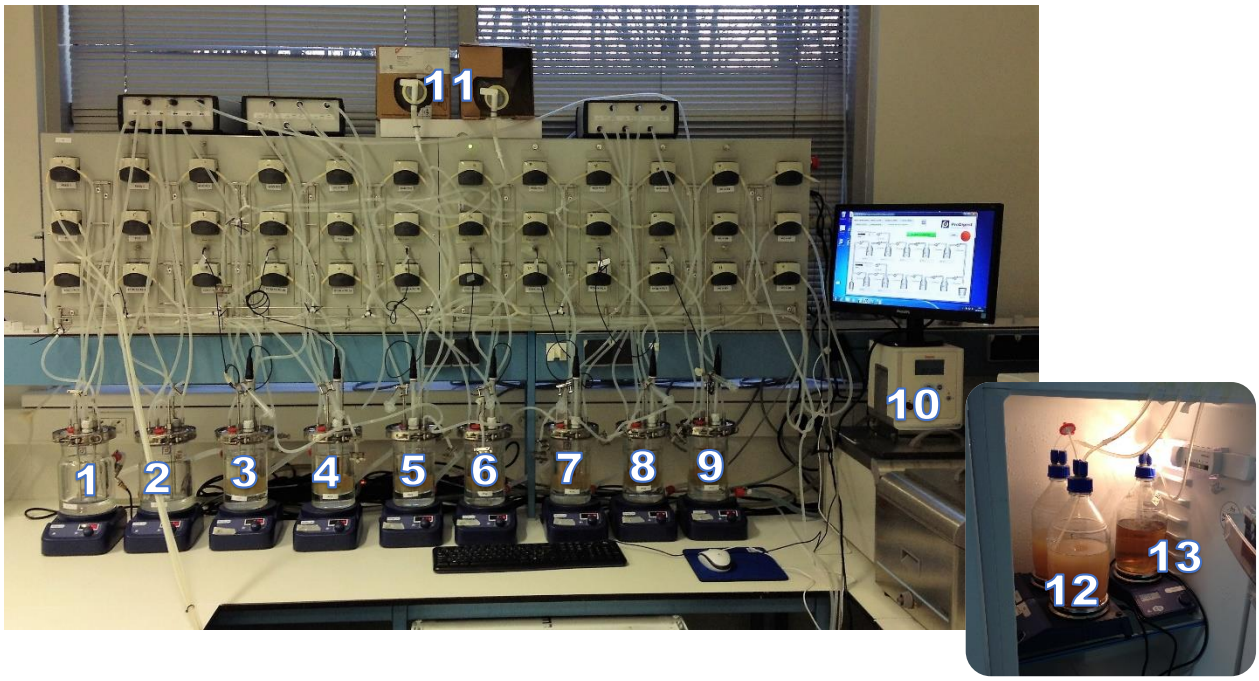


Figure 10 – Baby M-SHIME set-up used to grow bacterial communities. A computer controlled set-up of nine vessels was assembled. ***1,2 –*** stomachs/small intestines; ***3-9*** – proximal colons; ***10 –*** waterbath (37ºC); ***11 –*** HCl (0,5 M, Sigma Aldrich) and NaOH (0,5 M, Sigma Aldrich) soutions to control pH; ***12 –*** feed; ***13 –*** pancreatic juice.

The SHIME experiment consisted of the following phases:

- Start-up period, after inoculation of the colon reactors with faecal material from selected donor. This period allowed the microbial community to differentiate in the different reactors depending on the local environmental conditions. At the end of this period, two of the seven PC vessels were removed from the system since their conditions (SCFA, pH) were not similar to the rest.
- Control period, which allowed to determine the baseline microbial community composition and activity in the different reactors. This baseline was used as control to compare with the results from the prebiotic treatment. During this period, 0.1 g/L of the test products (GS and NMO) were added to both feed containers to prevent wash-out of microbes that are potentially specialized in degrading these compounds prior to the start of the 2-week treatment;
- Treatment period, in which the basic diet was amended with the specific test product(s) (3.2 g/L) to evaluate its impact on the composition and activity of the gut microbiota. In Table 5 are indicated the amounts GS and NMO in each PC.

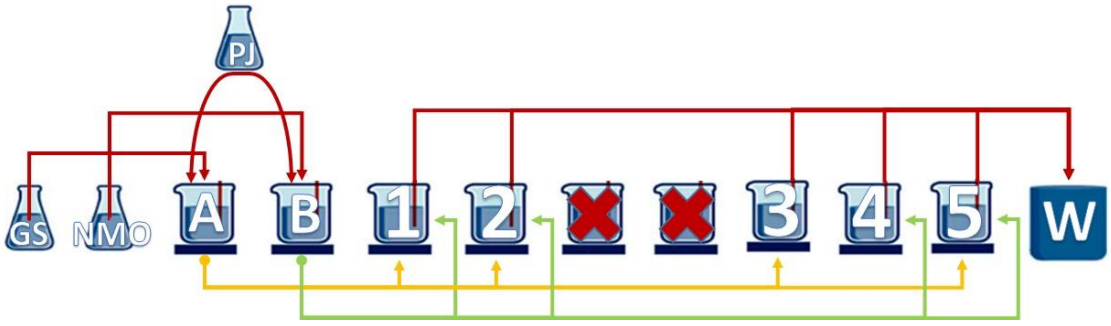


Figure 11 – Scheme of the baby M-SHIME set-up. ***GS* –** "Golden standard" feed (3.2 g/L); ***NMO* –** "New Milk Oligosaccharide" feed (3.2 g/L); ***PJ* –** pancreatic jJuice; ***A,B* –** stomachs/small intestines; ***1-5* –** proximal colons (other two vessels were excluded); ***W* –** waste container.

Table 5 – Percentage of each test product ("Golden Standard" and New Milk Oligosaccharide) on every PC.

PC	Feed	
	'Golden Standard'	*New Milk Oligosaccharide*
1	100 %	0 %
2	90 %	10 %
3	75 %	25 %
4	50 %	50 %
5	0 %	100 %

3. Metabolic Analysis

3.1 SCFAs

For the analysis of SCFA acetate, propionate and butyrate, the method described by De Weirdt et al. (2010) was applied. Briefly, SCFAs were extracted from the samples with diethyl ether, after the addition of 2-methyl hexanoic acid as an internal standard. Extracts were analysed using a GC-2014 gas chromatograph (Shimadzu®, 's-Hertogenbosch, Netherlands), equipped with a capillary fatty acid-free EC-1000 Econo-Cap column (Alltech®, Laarne, Belgium), a flame ionization detector and a split injector. The injection volume was 1 µL and the temperature profile was set from 110 to 160°C, with a temperature increase of 6 °C/min. The carrier gas was nitrogen and the temperature of the injector and detector were 100 and 220 °C, respectively.

3.2 Lactate

For the lactate analysis, a D-lactic/L-lactic acid kit (Bioline®, London, UK) was used. All the steps made were taken following the guidelines from the kit. At first, the absorbance (A1) of a mix of 50 µL of sample and 1060 µL of master mix was measured at 340 nm, using a UV-1800 spectrophotometer (Shimadzu). Subsequently, D-lactate dehydrogenase (D-LDH) and D-glutamate pyruvate transaminase (D-GPT), were added as a mixture (10 µL per sample) to induce the next reactions:

$$D-lactate + NAD^{+} \overset{D\text{-}LDH}{\leftrightarrow} pyruvate + NADH + H^{+}$$
$$pyruvate + D-glutamate \overset{D\text{-}GTP}{\leftrightarrow} D-alanine + 2-oxoglutarate$$

This two steps reaction consists in the conversion of D-lactate into pyruvate (1), which in turn is converted into D-alanine (2). After 30 minutes the absorbance (A2) is measured at 340 nm. In order to convert the remaining lactate (L-lactate), another 10 µL mixture of L-LDH and L-GTP was added to each sample. The following reactions occurred:

$$L-lactate + NAD^{+} \overset{L\text{-}LDH}{\leftrightarrow} pyruvate + NADH + H^{+}$$
$$pyruvate + L-glutamate \overset{L\text{-}GTP}{\leftrightarrow} L-alanine + 2-oxoglutarate$$

The absorbance (A3) was measured at 340 nm.

Total, D and L-lactate concentrations (g/L), c, were determined using the following equation:

$$c = \frac{V \times MW}{1000 \times \varepsilon \times d \times v} \Delta A$$

Where V is the final volume, v is the sample volume, MW is the molecular weight of lactate, d is the light path, ΔA is the difference in absorbance and ε is the extinction coefficient of NADH at 340 nm.

3.3 Ammonium

The ammonium analysis was performed using the FoodALYT D 5000 Steam Distillation Unit (Omnilab®, Bremen, Germany) complemented with Doorstroomkoeler Fryka DLK 602 cooling system (Voor‘t Labo®, Eeklo, Belgium) set at 10ºC. The ammonium was distilled in a slightly alkaline medium (MgO (ChemLab®, Zedelgem, Belgium)), in the form of ammonia and absorbed in 10 mL boric acid (Chem Lab) mixed indicator (pH 5.3). The ammonia in the distillate, now in the form of borate ($(NH_4)_3BO_3$) was determined titrimetrically with HCl (0.02 N) (VWR) using the Titroline (Omnilab).

The content of NH_4^+-N is calculated with following formula:

$$NH_4^+ - N\ \left(\frac{mg}{L}\right) = \frac{(A - B) \times 0{,}02 \times 14{,}01 \times 1000 \times f}{V_{sample}}$$

Where A is the volume of HCl titrated for the sample (mL), B is the volume of of HCl titrated for the blank (mL), V_{sample} is the volume of sample (mL) and f is the dilution factor.

4. Microbial Analysis

4.1 DNA Extraction

Total DNA was extracted using the Fast-Prep24 instrument (Mp-Bio®, Eschwege Germany), as previously described by Vilchez-Vargas et al. (2013). Briefly, for DNA extraction, the pellet of 1 mL of liquid sample or 0.25 g of mucus samples were re-suspended in 1 ml Tris/HCl (100 mM pH 8.0), supplemented with 100 mM EDTA, 100 mM NaCl, 1% (wt/vol) polyvinylpyrrolidone and 2% (wt/vol) sodium dodecyl sulphate and transferred to a 2 ml eppendorf tube containing 0.2 g beads (for the mechanical disruption). Cells were lysed in the Fast Prep-24 instrument (40 sec, 6.0 m/sec). Samples were centrifuged at 14000 G for 1 min at 4°C and the supernatant washed with one volume phenol/chloroform (1:1), centrifuged and the aqueous phase washed with one volume chloroform. After centrifugation, nucleic acids (aqueous phase) were precipitated with one volume of ice-cold isopropanol and 1:10 volume of 3 M sodium acetate. Next, the DNA was precipitated with three volumes of ethanol and 1:10 volume of 3 M sodium acetate. After centrifugation and washing with 80% ethanol the pellet was re-suspended in 50 ml of milliQ water. DNA quality and quantity were analysed electrophoretically on a 1% (w/v) agarose gels and spectrophotometrically by determination of the absorbance at 260 nm and the absorbance ratios at 260 nm and 280 nm, respectively.

4.2 Denaturing Gradient Gel Electrophoresis (DGGE)

DGGE profiling was used to monitor the most prominent shifts within the bifidobacterial microbial community. After DNA extraction and PCR with group-specific primers, DGGE was performed to separate PCR products (Table 5). Gels were run using a DCodeTM Universal Mutation Detection System (Bio-Rad®, Nazareth, Belgium) and data analysis was carried out using GelCompar version 6.6 (Applied Maths®, Sint-Martens-Latem, Belgium). Pearson correlation and UMPG clustering were used to calculate dendrograms of DGGE profiles.

Table 6 - PCR conditions and DGGE protocol for the investigated bacterial group.

Bacterial group	PCR primers	PCR protocol	DGGE protocol	Reference
Bifidobacteria *	BIF164f BIF662r	7' 95 °C; 35 x (1' 94 °C/1' 62 °C/2' 72 °C); 10' 72 °C	50-65%, 8%, 16h, 38V, 60°C	*(Satokari et al., 2001)*

* The PCR product of the group-specific PCR was diluted 1:100

4.3 Quantitative Polymerase Chain Reaction (qPCR)

A culture-independent method (qPCR) was applied to quantify Bifidobacteria. Specific primers, temperature conditions and primer concentrations can be found in Table 6. The PCR mixtures (total volume of 15 µL) contained 5µL template (between 1 -10 µg/µL DNA, corresponding to a 1:100 dilution of the original DNA extract) and 10µL QPCR SYBR Green ROX Mix (Westburg®, Leusden, Netherlands), forward and reverse primer (10 µM each). Negative controls for each batch of samples included template consisting of qPCR water. Samples were incubated in a StepOnePlus real-time PCR device (Applied Biosystems®, Bleiswijk, Netherlands). Each sample was analysed in triplicate. Samples were checked for correct peaks in the melt curve. The standard curve in all of the different runs should have an efficiency between 90-105%. If not, the run was repeated. Outliers (more than 1 CT difference) in triplicates were omitted. Resulting values were converted to copies/µL by multiplying with 10^2, i.e. by taking into account the dilution (there was a 1:100 dilution of DNA extract prior to the qPCR).

Table 7 - qPCR conditions for the investigated bacterial groups.

Bacterial group	PCR primers	PCR protocol	Primer (µM)
Bifidobacteria*	Bif243F (TCGCGTCYGGTGTGAAAG) Bif243R (CCACATCCAGCRTCCAC)	15' 95 °C; 40 x (1' 95 °C/1' 58 °C/1'45" 72 °C); 15" 95°C; 20" 70°C; 15" 95°C	0.5
Lactobacilli	F_Lacto_05 (AGCAGTAGGGAATCTTCCA) R_Lacto_04 (CGCCACTGGTGTTCYTCCATATA)	15' 95 °C; 40 x (15" 95 °C/1' 60°C); 15" 95°C; 20" 70°C; 15" 95°C	0.3
Firmicutes	Firm934F (GGAGYATGTGGTTTAATTCGAAGCA) Firm1060R (AGCTGACGACAACCATGCAC)	15' 95 °C; 40 x (15" 95 °C/1' 60°C); 15" 95°C; 20" 70°C; 15" 95°C	0.3
Bacteroidetes	Bact 934F (GGARCATGTGGTTTAATTCGATGAT) Bact 1060R (AGCTGACGACAACCATGCAG)	15' 95 °C; 40 x (15" 95 °C/1' 60°C); 15" 95°C; 20" 70°C; 15" 95°C	0.3
Enterobacteria	Ent-F (GTTGTAAAGCACTTTCAGTGGTGAGGAAGG) Ent-R (GCCTCAAGGGCACAACCTCCAAG)	15' 95 °C; 40 x (15" 95 °C/1' 60°C); 15" 95°C; 20" 70°C; 15" 95°C	0.3

* Remark = 1 µL MgCl2 were added to 1 mL of the original buffer

IV. BATCH EXPERIMENT

1. Results

Donors 1, 3, 6 and 8

Tables 7, 8, 9 and 10 present the pH and SCFA values of control and treatment at 0h, 24h and 48h after inoculation, for donors 1, 3, 6 and 8, respectively. All of them indicate a decreased pH over time with both treatment and control, though more pronounced with treatment. The four donors showed a high increase of acetate with the treatment. Further, while in donors 1 and 3 the propionate increase was not significant even with treatment, donors 6 and 8 showed the highest amounts among all the 10 donors. Butyrate concentrations resulting from treatment were generally very low.

Figures 10, 11, 12 and 13 display the quantitative and qualitative alterations in the Bifidobacteria population of each donor. By comparing inoculum with 48h bottles, a higher growth was observed when the treatment was applied, although no alterations were observed in the microbiotas' structure as assessed with DGGE. Donor 6 was the only exception, showing a few differences in terms of abundance of some strains.

Table 8 – Average pH and SCFAs' concentrations (± SD) (mM) obtained at different time-points (0h, 24h, 48h) from inoculum, control and treatment bottles of the batch experiment using faecal material of donor **baby 1**.

	pH		**Acetic Acid**		**Propionic Acid**		**Butyric Acid**	
	Ctrl	Treat	Ctrl	Treat	Ctrl	Treat	Ctrl	Treat
0h	6.36	6.34 ± *0.01*	0.6		0.0		0.0	
24h	6.09	4.48 ± *0.00*	7.5	22.6 ± *0.3*	2.5	0.0 ± *0.0*	0.0	0.0 ± *0.0*
48h	6.19	4.39 ± *0.00*	10.2	25.2 ± *0.3*	3.4	0.0 ± *0.0*	0.8	0.0 ± *0.0*

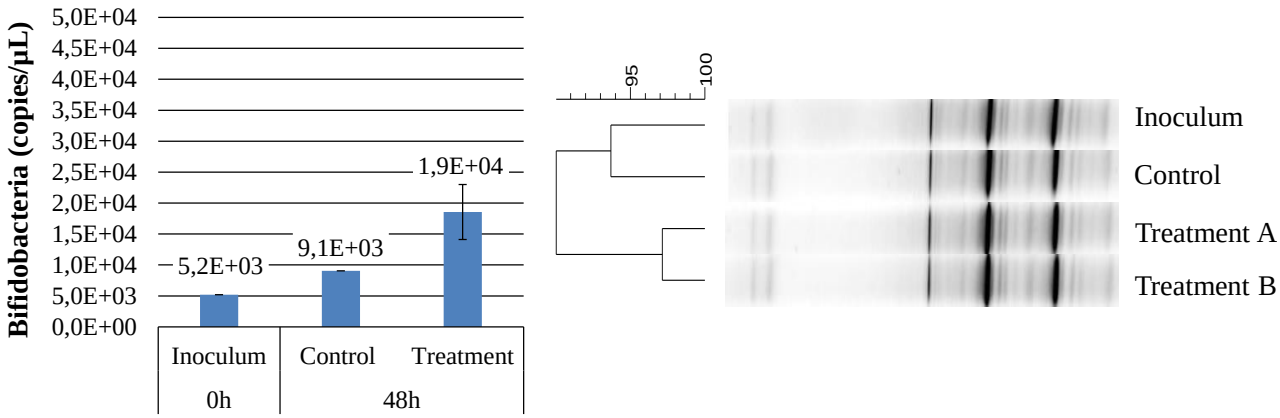


Figure 12 – Absolute Bifidobacteria numbers (left: copies/µL) and qualitative changes within the bifidobacterial community (right) at the beginning and end of the incubation (48h), both for the control and the treatment. Values were obtained from a batch experiment using faecal material of donor **baby 1**.

Table 9 – Average pH and SCFAs' concentrations (± SD) (mM) obtained at different time-points (0h, 24h, 48h) from inoculum, control and treatment bottles of the batch experiment using faecal material of donor **baby 3**.

	pH		**Acetic Acid**		**Propionic Acid**		**Butyric Acid**	
	Ctrl	Treat	Ctrl	Treat	Ctrl	Treat	Ctrl	Treat
0h	6.35	6.30 ± *0.03*	1.8		0.0		0.0	
24h	5.92	4.47 ± *0.00*	8.1	28.7 ± *0.24*	0.2	0.0 ± *0.0*	0.0	0.0 ± *0.0*
48h	6.11	4.44 ± *0.01*	8.8	29.2 ± *0.38*	0.3	0.0 ± *0.0*	0.0	0.0 ± *0.0*

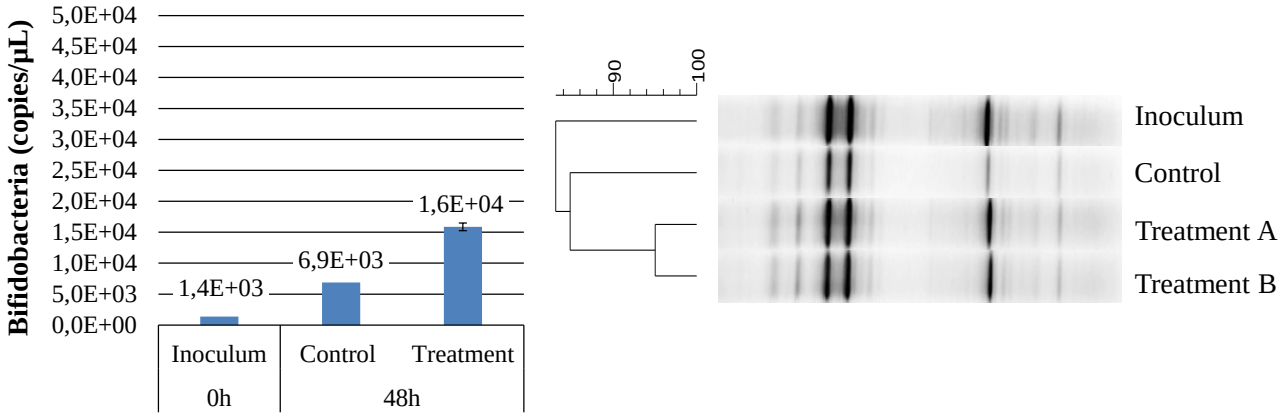


Figure 13 – Absolute Bifidobacteria numbers (left: copies/µL) and qualitative changes within the bifidobacterial community (right) at the beginning and end of the incubation (48h), both for the control and the treatment. Values were obtained from a batch experiment using faecal material of donor **baby 3**.

Table 10 – Average pH and SCFAs' concentrations (± SD) (mM) obtained at different time-points (0h, 24h, 48h) from inoculum, control and treatment bottles of the batch experiment using faecal material of donor **baby 6**.

	pH		**Acetic Acid**		**Propionic Acid**		**Butyric Acid**	
	Ctrl	Treat	Ctrl	Treat	Ctrl	Treat	Ctrl	Treat
0h	6.44	6.37 ± *0.02*	0.6		0.2		0.1	
24h	6.17	4.76 ± *0.08*	8.8	20.1 ± *0.4*	3.1	18.6 ± *0.2*	1.7	0.1 ± *0.0*
48h	6.32	4.91 ± *0.00*	9.8	20.7 ± *0.1*	3.4	18.6 ± *0.0*	2.0	0.1 ± *0.0*

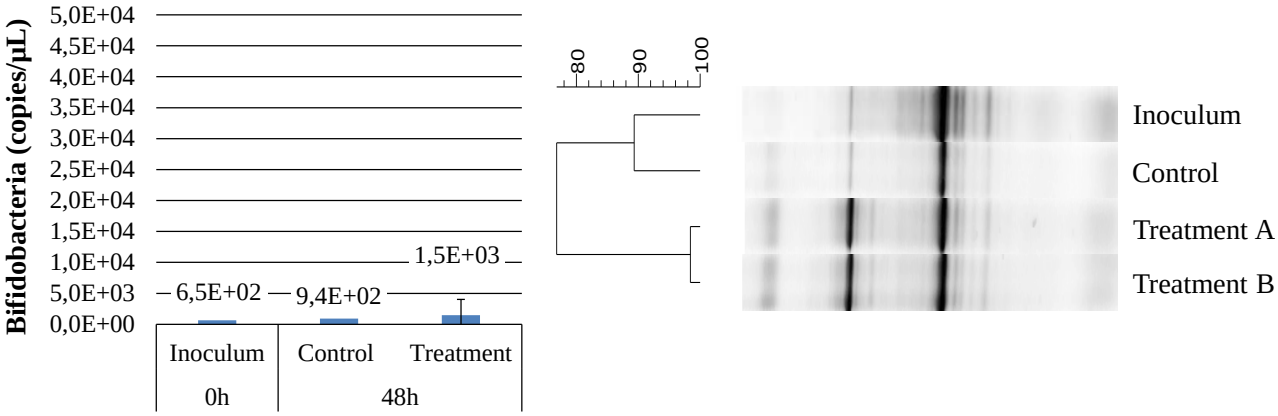


Figure 14 – Absolute Bifidobacteria numbers (left: copies/µL) and qualitative changes within the bifidobacterial community (right) at the beginning and end of the incubation (48h), both for the control and the treatment. Values were obtained from a batch experiment using faecal material of donor **baby 6**.

Table 11 – Average pH and SCFAs' concentrations (± SD) (mM) obtained at different time-points (0h, 24h, 48h) from inoculum, control and treatment bottles of the batch experiment using faecal material of donor **baby 8**.

	pH		**Acetic Acid**		**Propionic Acid**		**Butyric Acid**	
	Ctrl	Treat	Ctrl	Treat	Ctrl	Treat	Ctrl	Treat
0h	6.44	6.38 ± *0.01*	1.0		0.1		0.1	
24h	6.14	4.97 ± *0.00*	8.7	27.5 ± *1.2*	2.0	7.2 ± *0.3*	0.9	0.2 ± *0.0*
48h	6.15	4.99 ± *0.00*	11.4	26.6 ± *0.1*	2.1	7.3 ± *0.1*	2.0	0.2 ± *0.0*

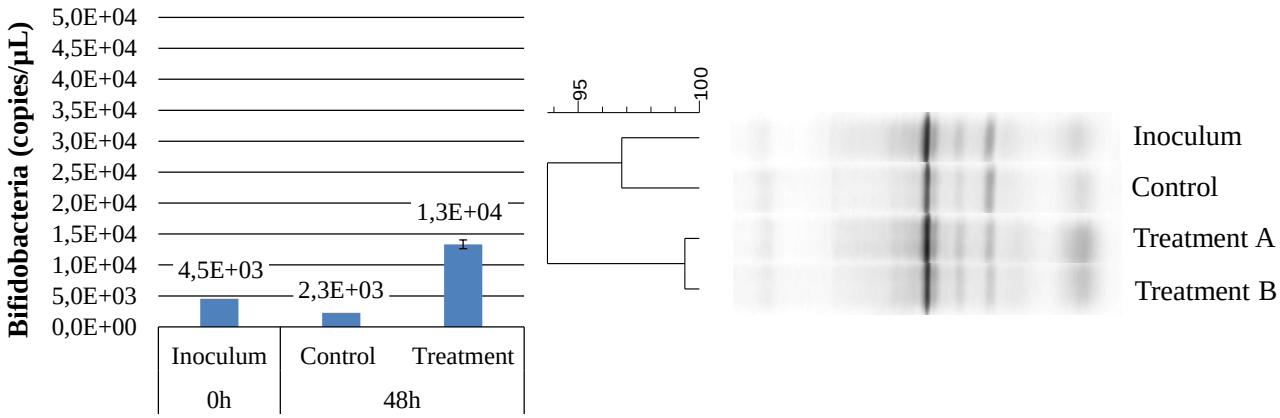


Figure 15 – Absolute Bifidobacteria numbers (left: copies/µL) and qualitative changes within the bifidobacterial community (right) at the beginning and end of the incubation (48h), both for the control and the treatment. Values were obtained from a batch experiment using faecal material of donor **baby 8**.

Donors 2, 4 and 5

Tables 11, 12 and 13 present the pH and SCFA values of the control and treatment at 0h, 24h and 48h after incubation, for donors 2, 4 and 5, respectively. All of them show a small decrease in pH over time in both treatment and control. When compared with the rest of the donors, these three donors showed low increases in acetate, propionate and butyrate upon dosing the treatment.

Figures 14, 15 and 16 display the quantitative and qualitative alterations in the Bifidobacteria population of each donor. While donors 4 and 5 had a very low number of Bifidobacteria in the inoculum, control and treatment, donor 2 even revealed a decrease over time. No alterations were observed in the microbiota's composition of the three donors.

Table 12 – Average pH and SCFAs' concentrations (± SD) (mM) obtained at different time-points (0h, 24h, 48h) from inoculum, control and treatment bottles of the batch experiment using faecal material of donor **baby 2**.

	pH		**Acetic Acid**		**Propionic Acid**		**Butyric Acid**	
	Ctrl	Treat	Ctrl	Treat	Ctrl	Treat	Ctrl	Treat
0h	6.38	6.30 ± *0.01*	0.3		0.0		0.0	
24h	6.00	6.09 ± *0.08*	4.8	5.8 ± *0.0*	1.4	1.8 ± *0.0*	0.5	0.4 ± *0.0*
48h	6.29	6.08 ± *0.08*	7.2	10.8 ± *0.2*	1.7	3.1 ± *0.0*	1.4	1.2 ± *0.0*

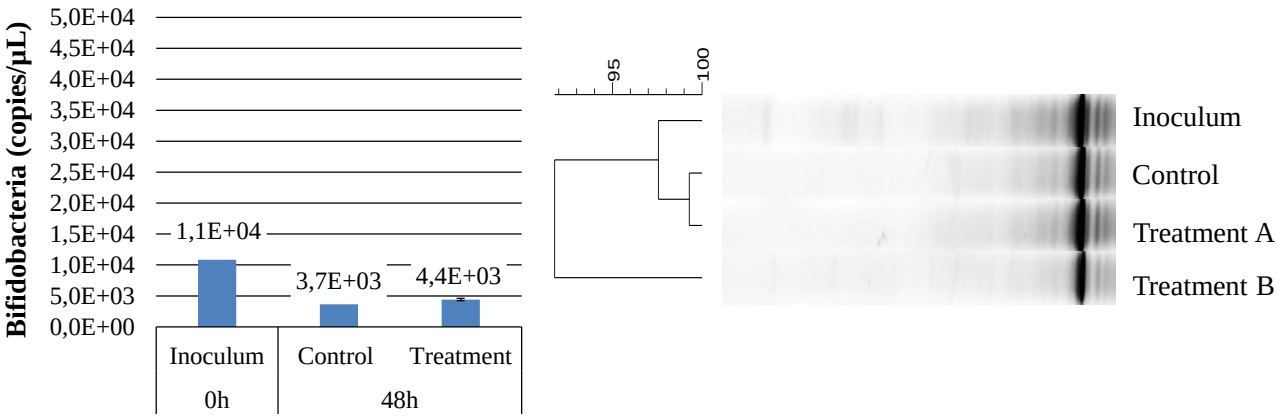


Figure 16 – Absolute Bifidobacteria numbers (left: copies/µL) and qualitative changes within the bifidobacterial community (right) at the beginning and end of the incubation (48h), both for the control and the treatment. Values were obtained from a batch experiment using faecal material of donor **baby 2**.

Table 13 – Average pH and SCFAs' concentrations (± SD) (mM) obtained at different time-points (0h, 24h, 48h) from inoculum, control and treatment bottles of the batch experiment using faecal material of donor **baby 4**.

	pH		**Acetic Acid**		**Propionic Acid**		**Butyric Acid**	
	Ctrl	Treat	Ctrl	Treat	Ctrl	Treat	Ctrl	Treat
0h	6.38	6.37 ± *0.02*	0.5		0.1		0.0	
24h	6.27	6.21 ± *0.01*	5.5	7.0 ± *0.0*	1.3	1.7 ± *0.0*	1.1	1.2 ± *0.0*
48h	6.34	6.18 ± *0.04*	7.1	10.3 ± *0.1*	1.5	2.4 ± *0.0*	1.5	1.5 ± *0.0*

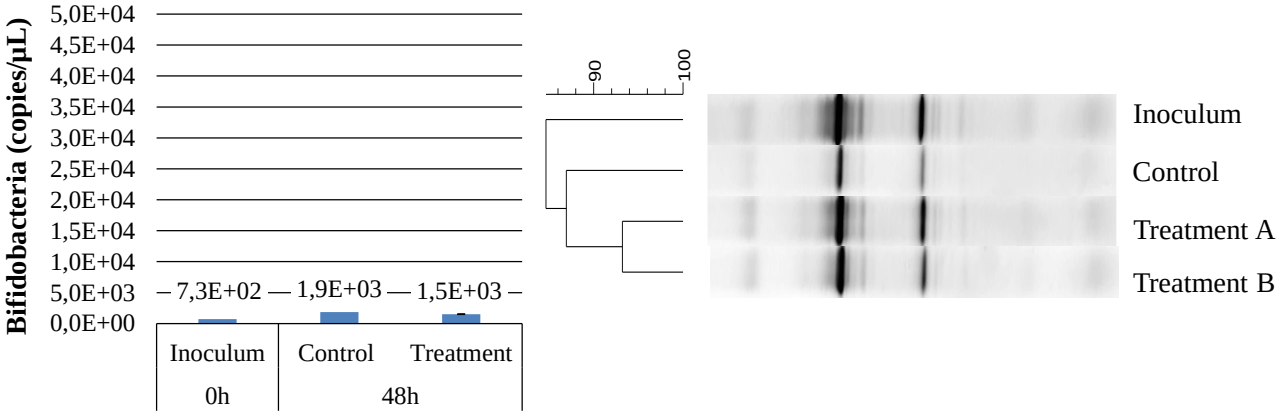


Figure 17 – Absolute Bifidobacteria numbers (left: copies/µL) and qualitative changes within the bifidobacterial community (right) at the beginning and end of the incubation (48h), both for the control and the treatment. Values were obtained from a batch experiment using faecal material of donor **baby 4**.

Table 14 – Average pH and SCFAs' concentrations (± SD) (mM) obtained at different time-points (0h, 24h, 48h) from inoculum, control and treatment bottles of the batch experiment using faecal material of donor **baby 5**.

	pH		**Acetic Acid**		**Propionic Acid**		**Butyric Acid**	
	Ctrl	Treat	Ctrl	Treat	Ctrl	Treat	Ctrl	Treat
0h	6.28	6.39 ± *0.02*	0.1		0.0		0.0	
24h	6.23	6.14 ± *0.02*	3.6	4.1 ± *0.1*	1.7	2.0 ± *0.0*	0.0	0.0 ± *0.0*
48h	6.29	6.27 ± *0.00*	3.9	5.0 ± *0.1*	2.4	3.6 ± *0.0*	0.0	0.0 ± *0.0*

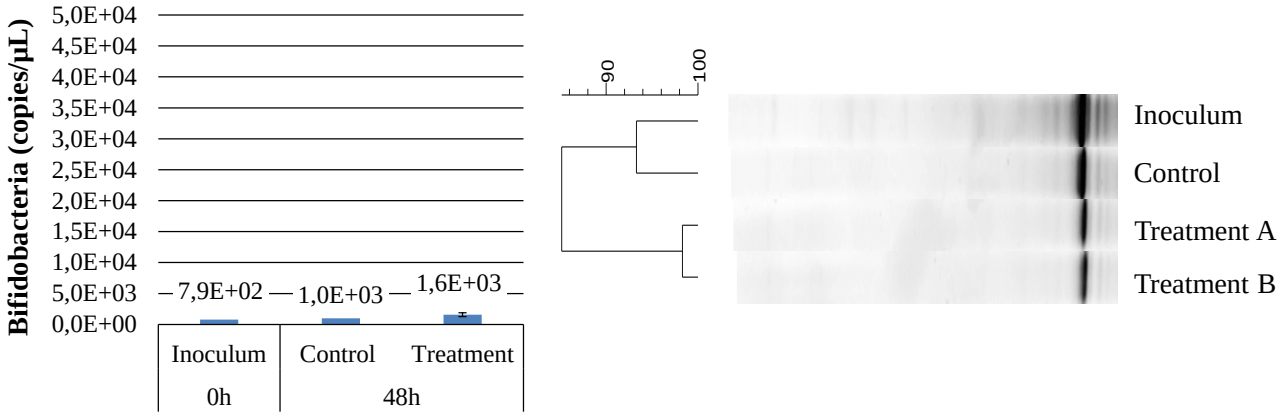


Figure 18 – Absolute Bifidobacteria numbers (left: copies/µL) and qualitative changes within the bifidobacterial community (right) at the beginning and end of the incubation (48h), both for the control and the treatment. Values were obtained from a batch experiment using faecal material of donor **baby 5**.

Donors 7, 9 and 10

Tables 14, 15 and 16 present the pH and SCFA values of the control and treatment at 0h, 24h and 48h after incubation, for donors 7, 9 and 10, respectively. These three donors show a decrease in pH over time with both treatment and control, though more pronounced with treatment. They all showed increases of acetate, propionate and butyrate concentrations upon dosing the treatment. The most relevant value is the butyrate concentration of donor 10, which reached 3.9 mM after 48 h, the highest between all donors.

Figures 17, 18 and 19 display quantitative and qualitative alterations in the Bifidobacteria population of each donor. These three donors showed the highest increase of Bifidobacteria or presented the largest differences treatment *vs* inoculum. Donor 10 was the only one showing alterations in the bifidobacterial composition, with a specific increase of a band that was identified as *Bifidobacterium longum* spp. i*nfantis*.

Table 15 – Average pH and SCFAs' concentrations (± SD) (mM) obtained at different time-points (0h, 24h, 48h) from inoculum, control and treatment bottles of the batch experiment using faecal material of donor **baby 7.**

	pH		**Acetic Acid**		**Propionic Acid**		**Butyric Acid**	
	Ctrl	Treat	Ctrl	Treat	Ctrl	Treat	Ctrl	Treat
0h	6.44	6.47 ± *0.01*	0.9		0.1		0.2	
24h	NA	4.68 ± *0.00*	NA	34.1 ± *0.2*	NA	4.8 ± *0.0*	NA	2.0 ± *0.0*
48h	NA	4.67 ± *0.04*	NA	31.9 ± *0.3*	NA	4.5 ± *0.0*	NA	1.9 ± *0.0*

(Note: Control values at 24h and 48h were not possible to obtain)

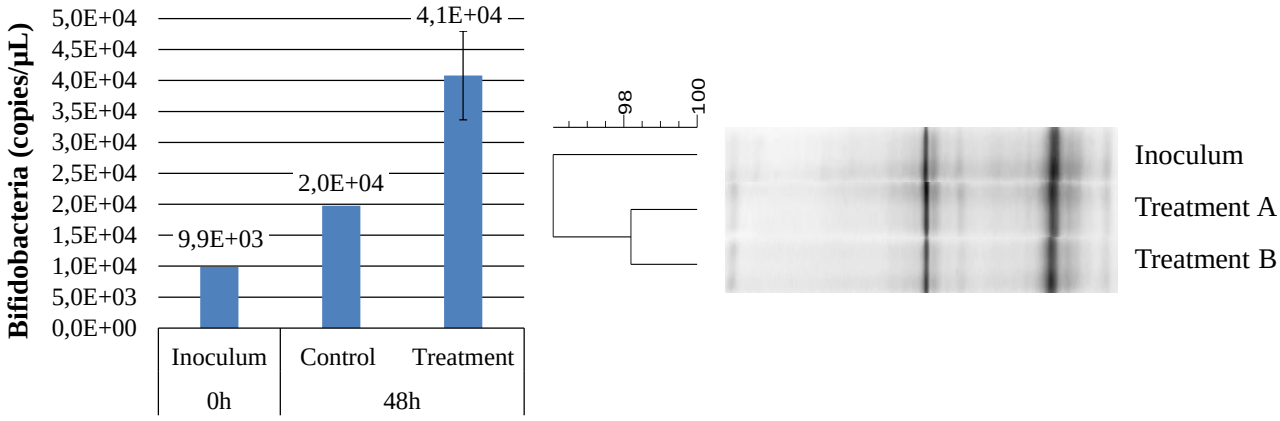


Figure 19 – Absolute Bifidobacteria numbers (left: copies/µL) and qualitative changes within the bifidobacterial community (right) at the beginning and end of the incubation (48h), both for the control and the treatment. Values were obtained from a batch experiment using faecal material of donor **baby 7**.

Table 16 – Average pH and SCFAs' concentrations (± SD) (mM) obtained at different time-points (0h, 24h, 48h) from inoculum, control and treatment bottles of the batch experiment using faecal material of donor ***baby 9*.**

	pH		**Acetic Acid**		**Propionic Acid**		**Butyric Acid**	
	Ctrl	Treat	Ctrl	Treat	Ctrl	Treat	Ctrl	Treat
0h	6.39	6.39 ± *0.02*	0.7		0.1		0.1	
24h	6.16	4.43 ± *0.02*	10.8	28.7 ± *0.9*	5.1	4.5 ± *0.2*	1.3	0.2 ± *0.0*
48h	6.23	4.48 ± *0.01*	10.1	27.7 ± *0.2*	5.4	4.9 ± *0.0*	1.8	0.2 ± *0.0*

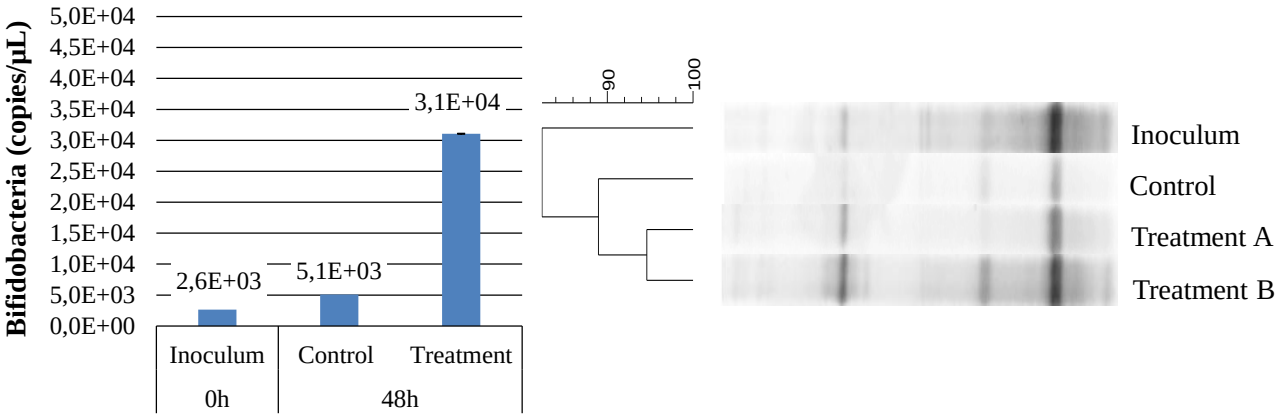


Figure 20 – Absolute Bifidobacteria numbers (left: copies/µL) and qualitative changes within the bifidobacterial community (right) at the beginning and end of the incubation (48h), both for the control and the treatment. Values were obtained from a batch experiment using faecal material of donor **baby 9**.

Table 17 – Average pH and SCFAs' concentrations (± SD) (mM) obtained at different time-points (0h, 24h, 48h) from inoculum, control and treatment bottles of the batch experiment using faecal material of donor **baby 10.**

	pH		**Acetic Acid**		**Propionic Acid**		**Butyric Acid**	
	Ctrl	Treat	Ctrl	Treat	Ctrl	Treat	Ctrl	Treat
0h	6.41	6.42 ± *0.03*	0.1		0.0		0.0	
24h	5.62	5.49 ± *0.00*	5.3	7.0 ± *0.2*	1.6	1.7 ± *0.1*	0.9	3.3 ± *0.2*
48h	5.84	4.28 ± *0.00*	6.7	22.7 ± *0.5*	2.8	2.8 ± *0.0*	1.6	3.9 ± *0.1*

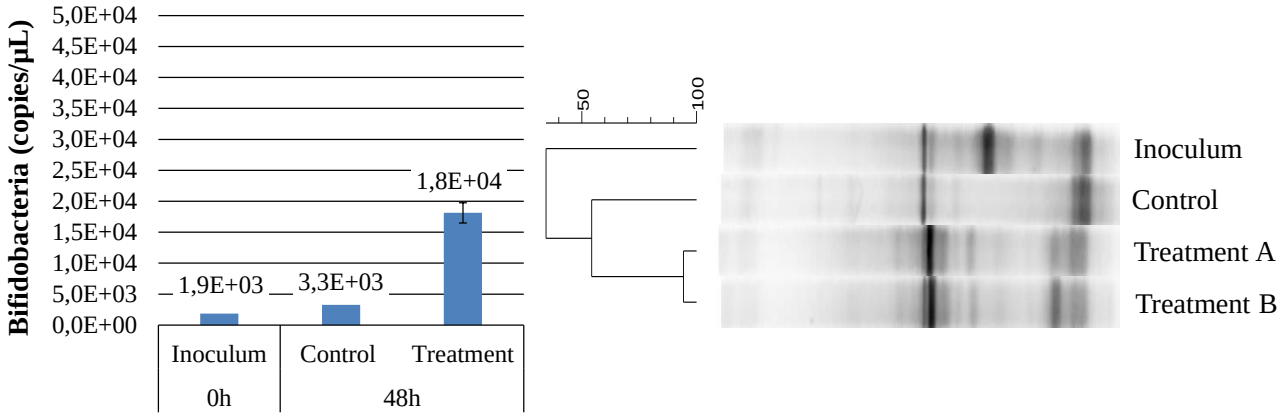


Figure 21 – Absolute Bifidobacteria numbers (left: copies/µL) and qualitative changes within the bifidobacterial community (right) at the beginning and end of the incubation (48h), both for the control and the treatment. Values were obtained from a batch experiment using faecal material of donor **baby 10**.

2. Discussion

For the selection of one donor out of the ten donors, results were analysed and compared to choose the one with the best response to treatment.

pH

This feature gave a first insight in the overall bacterial growth/activity during the incubations, since the latter results in a decreased pH due to the accumulation of produced acids, such as SCFAs.

Figure 20 provides an overview of the pH differences after 24 and 48 hours, between the inoculum and both the treatment and the control, for all the 10 donors. After 48 hours, all donors responded with decreased pH, although not relevant in case of donors 2, 4 and 5. On the other hand, donors 1, 3, 6, 7, 8, 9 and 10 showed an appreciable decrease. Donor 10 had the lowest pH and was the only one who presented a clear decrease after 24 hours indicating a slower initial degradation. This can be either because the inoculum contained less bacteria or bacteria responsible for breakdown were less abundant, requiring more time to grow. Then again, if we compare the control results of donor 10 with the other lower controls we can also suggest that this donor had a much richer microbiota. This could have been known if a full analysis of the microbiota present in the inocculum had been done.

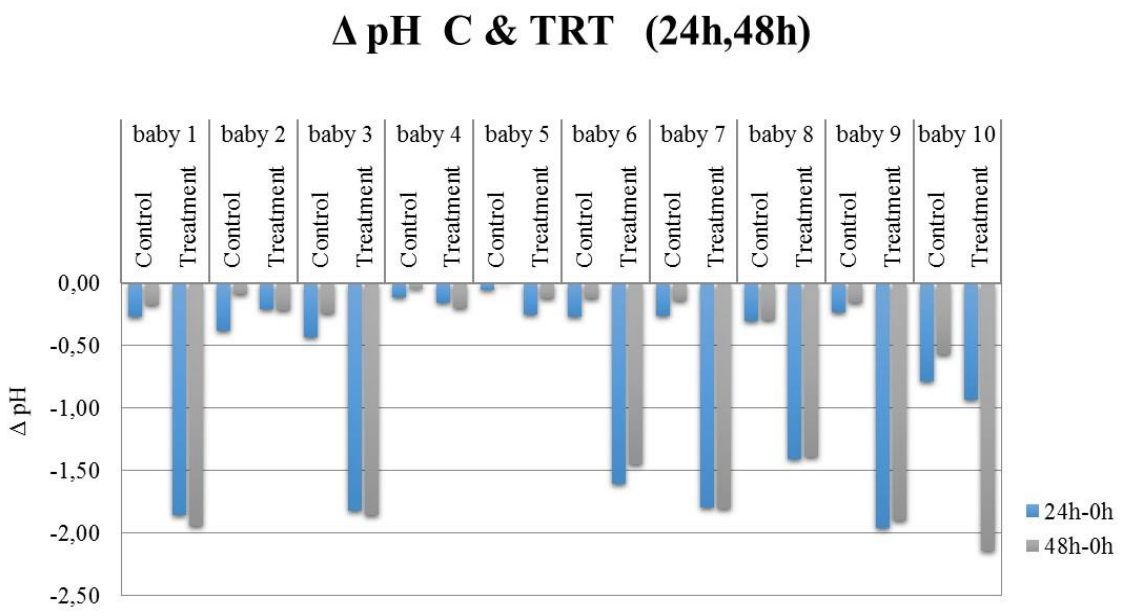


Figure 22 - pH differences between the inoculum and both treatment and control (24h and 48h bottles) obtained from batch experiment. *(Note: control values of baby 7 are averages of control values of all the other donors.)*

SCFAs

The concentrations of each SCFA were also determined concerning the metabolic activity of bacteria. Figure 21 shows the overview of the difference between treatment and control after 48 hours. First, point out that all donors, except 2, 4 and 5, showed high production of acids, proving a good correlation with the aforementioned pH results. The acetate production was very similar among the different donors tested, reaching higher values when the treatment was applied, with improvements ranging from around 10 mM up to almost 25 mM. With respect to propionate, seven out of ten donors also had better acids production with the treatment, from which donors 6 and 8 stand out (around 15 and 5 mM, respectively). Finally, donors 7 and 10 showed an enhancement in butyrate production with treatment, increasing 2.24 mM over control in the latter. As already mentioned, the production of butyrate by a microbiota can be linked to presence of Bifidobacteria due to the process of cross-feeding.

As a remark, applying the treatment to donors 7, 6 and 10 resulted in the highest concentrations of acetate (31.9 mM), propionate (18.6 mM) and butyrate (3.9 mM), respectively.

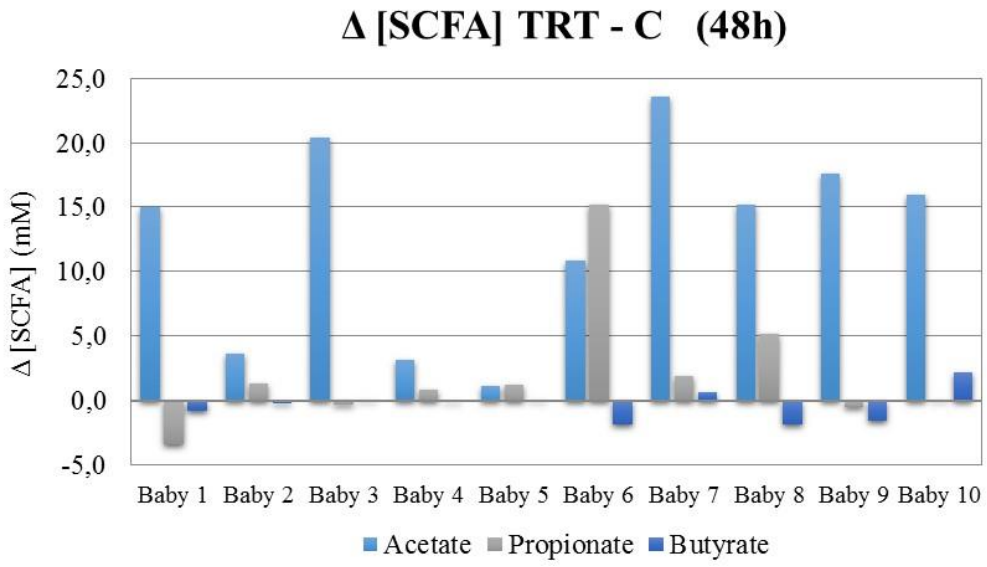


Figure 23 – Differences of acetate, propionate and butyrate concentrations between treatment and control 48h bottles. Values were obtained in a batch experiment. *(Note: For the control values of baby 7, an average of all the other control bottles was used)*

Bifidobacteria

Finally, the bifidobacterial community was analysed quantitatively and qualitatively. Figure 22 shows the overview of the difference of the absolute number of Bifidobacteria between treatment and control after 48 hours. All donors, except 2, 4 and 5, indicated higher growth in treatment bottles, with donors 7 and 9 showing the most marked increase, followed by donors 8 and 10. The first group obtained a difference between 2.5E04 and 3.0E04 copies/µL, while the latter had a difference between 1.0E04 and 1.5E04 copies/µL.

With respect to the bifidobacterial composition, donor 10 was the only donor showing differences between the treatment and the control that were observable in the DGGE pictures. Although sequencing and identification have yet to be done, from previous projects of ProDigest we know that the new central darker band appearing with treatment (Figure 19) is due to the presence of *Bifidobacterium longum* spp. *infantis*, a common species in a healthy breast-fed infants' gut microbiota.

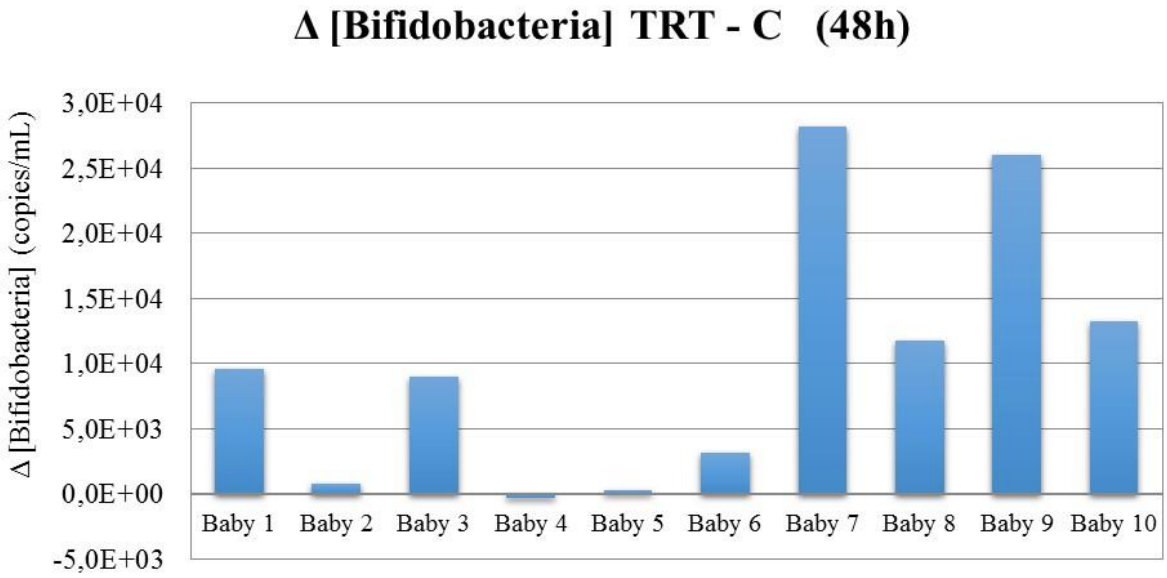


Figure 24 – Difference of the bifidobacterial concentration (copies/mL) between treatment and control 48h bottles. Values were obtained from a batch experiment.

B. infantis was already shown to have a 43-kb gene cluster (HMO cluster I) encoding a variety of oligosaccharide transport proteins, glycoside hydrolases (fucosidases, sialidases, β-hexosaminidase and β-galactosidase) and permeases, all predicted to hydrolyse and internalize human milk-derived oligosaccharides thereby generating monosaccharides (Ventura et al., 2015). This gene cluster is not found in other bifidobacterial species.

Moreover, only *B. infantis*, the archetypical HMO-utilizing bacterium, is able to digest all HMO structures (Underwood et al., 2015).

When grown in the presence of HMOs, *B. infantis* strains upregulate the expression of the gene encoding for the oligosaccharides transport proteins that bind to specific HMO linkages. The same does not happen when an identic strain is grown on the simpler prebiotic oligosaccharides FOS or GOS, suggesting that *B. infantis* is able to transport intact HMOs into its cytoplasm and that this capacity is "turned on" by the HMOs (Underwood et al., 2015).

B. infantis protective mechanisms are based on its anti-inflammatory and intestinal permeability decrease properties, SCFA production and also its increased colonization resulting in decreased diversity of the gut microbiota and fewer luminal pathogens (Underwood et al., 2015). Furthermore, positive correlations between *B. infantis* infant gut predominance and better weight gain and better responses to oral polio, tuberculosis, and tetanus vaccines have already been demonstrated. Still, this correlation does not determine the cause and consequence (Underwood et al., 2015).

In conclusion, **donor 10** was selected to proceed with this study taking into account the effects caused by the new treatment, namely: the decreasing pH, the increase of SCFAs concentration, especially butyrate, and the alterations of the bifidobacterial population with the emergence of *B. infantis*.

V. BABY M-SHIME

1. Results

Base-Acid Consumption

To make sure that optimal environmental conditions were maintained, the pH in the baby M-SHIME® system was controlled by pH controllers between 5.4-5.6 in the proximal colons (PCs) and the adding of acid and/or base.

Figure 25 presents the base-acid consumption (mL) per day of every PC (100/0, 90/10, 75/25, 50/50 and 0/100 of GS/NMO) during control (C1, 2 and 3) and treatment weeks (T1, 2, 3, 4, 5 and 6). This graph provides a clear view of the development of this parameter over time. All the 5 PCs indicate a stable and similar control week, as expected, and a pronounced increase when starting the treatment, at T1. Every PC reaches its peak at T3 (end of the 1st treatment week).

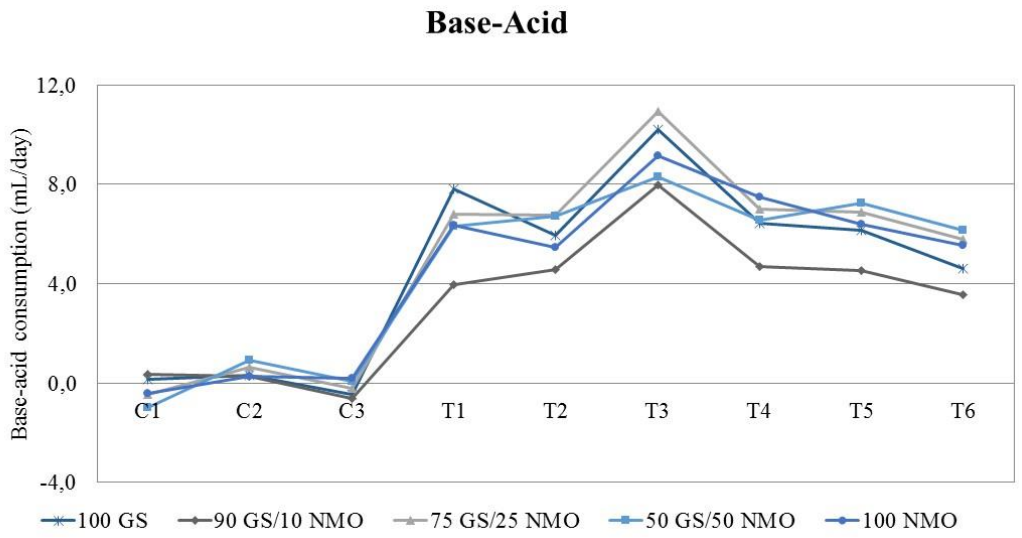
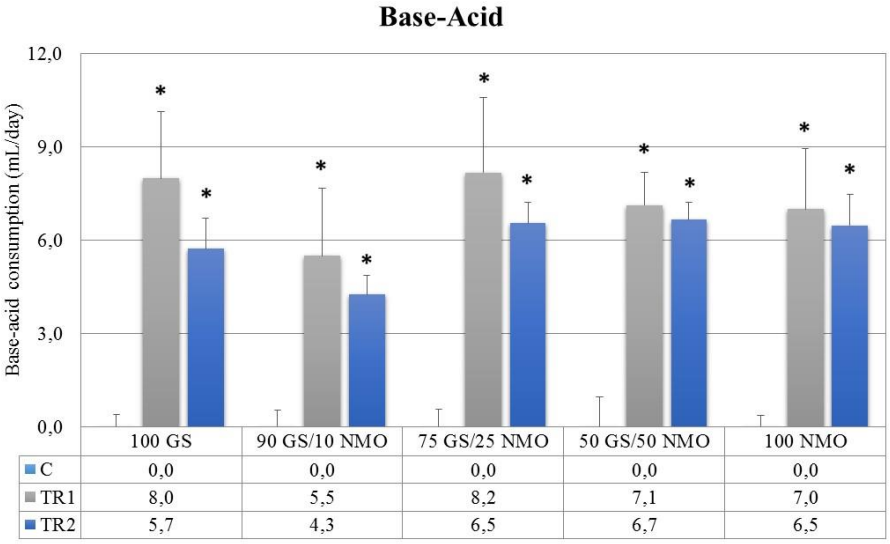


Figure 25 - Base-acid consumption (mL) during the control and treatment periods (100/0, 90/10, 75/25, 50/50 and 0/100 of GS/NMO), in the proximal colon (PC) of the baby M-SHIME, inoculated with faecal material of baby 10 (n = 3).
Note: Control values were normalized.

Figure 26 displays the average amount of acid and base consumed (mL) per week. Like the previous figure, this graph shows a large increase from control to 1st treatment week, for all units. On the other hand, during the 2nd treatment week a slight decrease is observed. All units' base-acid consumptions were very similar excepting for 90GS/10NMO

which showed lower values than the rest. Nonetheless, every single PC had a significant increase during treatment weeks in comparison with control.

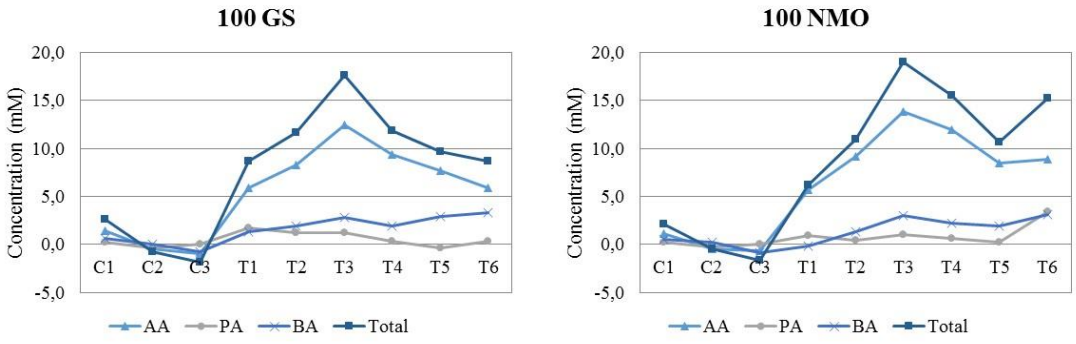


	100 GS	90 GS/10 NMO	75 GS/25 NMO	50 GS/50 NMO	100 NMO
C	0,0	0,0	0,0	0,0	0,0
TR1	8,0	5,5	8,2	7,1	7,0
TR2	5,7	4,3	6,5	6,7	6,5

Figure 26 – Average base-acid consumptions (mL) during the control and treatment weeks (100/0, 90/10, 75/25, 50/50 and 0/100 of GS/NMO), in the proximal colon (PC) of the baby M-SHIME, inoculated with faecal material of baby 10 (n = 3).
** : difference against control statistically significant (p-value < 0.05).*

SCFAs

Such as in the batch experiment, the concentrations of each SCFA were also determined concerning the metabolic activity of bacteria. Figure 27 shows the absolute variance of acetate, propionate, butyrate and total SCFAs concentrations (mM) over time. Every PC showed an increase of each SCFA after control week and, like the base-acid consumption, peaks were reached at T3 (end of 1st treatment week). Noteworthy, the fact that 100NMO got the highest peak of total SCFAs, close to 20 mM, at T3.



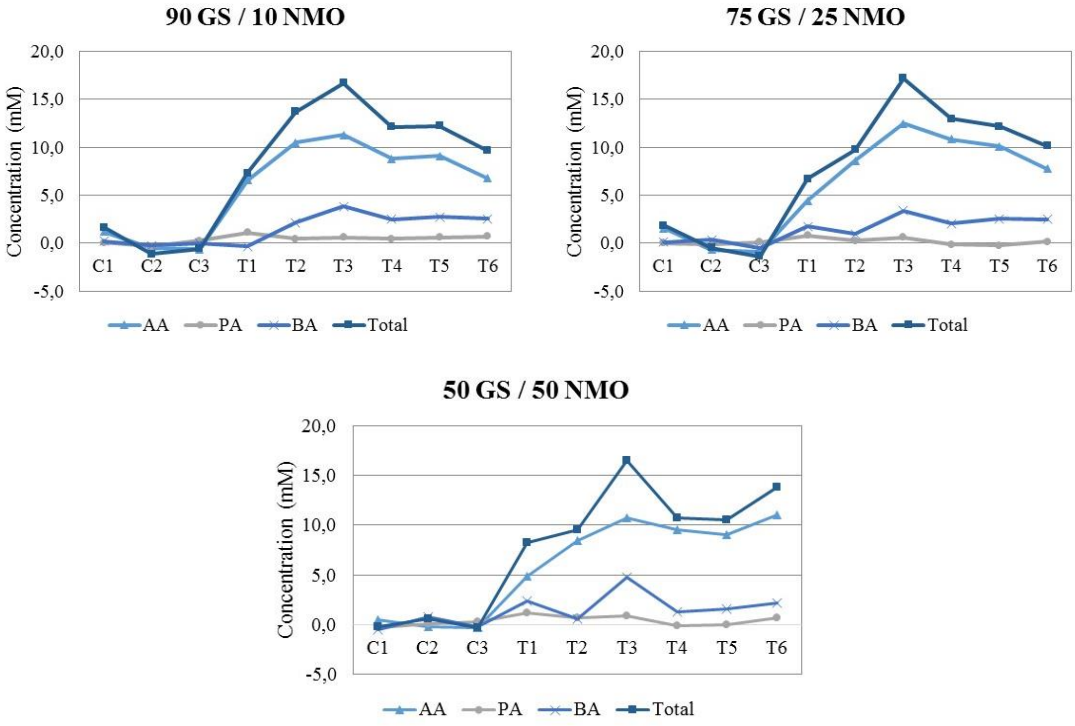
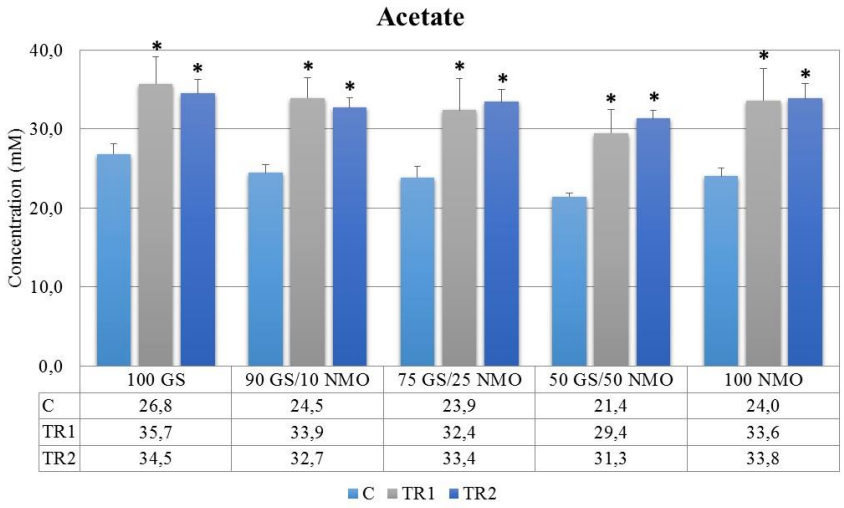


Figure 27 - Acetate, Propionate, Butyrate and total SCFA production during the control and treatment period (100/0, 90/10, 75/25, 50/50 and 0/100 of GS/NMO), in the proximal colon (PC) of the baby M-SHIME, inoculated with faecal material of baby 10. Graphs represent the absolute increase in concentration as compared to the average level during the control period (mM). *Note: Control values were normalized.*

The following figures display the average amount of each SCFA (mM) per week.

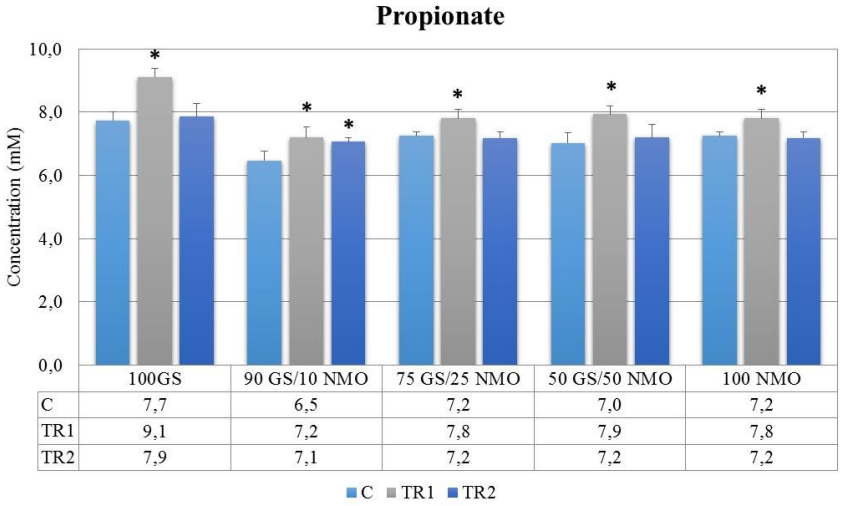
Acetate levels (mM), presented in Figure 28, significantly increased during both treatment weeks. Similar to the pre-screening, where mainly acetate increased upon NMO treatment, also during the SHIME experiment, acetate was the SCFA that most strongly increased upon GS/NMO treatments (~10mM increase vs control). All PCs had significant increases during in both treatment weeks, always reaching values above 30 mM.



	100 GS	90 GS/10 NMO	75 GS/25 NMO	50 GS/50 NMO	100 NMO
C	26,8	24,5	23,9	21,4	24,0
TR1	35,7	33,9	32,4	29,4	33,6
TR2	34,5	32,7	33,4	31,3	33,8

Figure 28 - Acetate levels (mM) during the control and treatment period (100/0, 90/10, 75/25, 50/50 and 0/100 of GS/NMO), in the proximal colon (PC) of the baby M-SHIME, inoculated with faecal material of baby 10 (n = 3).
** : difference against control statistically significant (p-value < 0.05).*

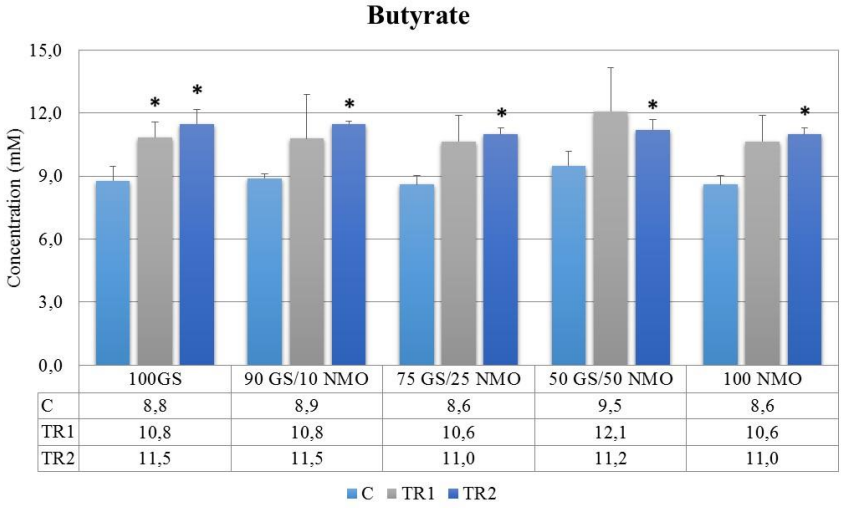
As for **propionate** levels (mM), exposed in Figure 29, a slight increase was registered during the 1st treatment week (~0.5-1.5 mM increase vs control), although this effect was temporary as it was not observed during the 2nd treatment week. The highest value, specifically 9.1 mM, was obtained by the 100GS during the 1st treatment week. All the others were very close to 8.0 mM.



	100GS	90 GS/10 NMO	75 GS/25 NMO	50 GS/50 NMO	100 NMO
C	7,7	6,5	7,2	7,0	7,2
TR1	9,1	7,2	7,8	7,9	7,8
TR2	7,9	7,1	7,2	7,2	7,2

Figure 29 - Propionate levels (mM) during the control and treatment period (100/0, 90/10, 75/25, 50/50 and 0/100 of GS/NMO), in the proximal colon (PC) of the baby M-SHIME, inoculated with faecal material of baby 10 (n = 3).
** : difference against control statistically significant (p-value < 0.05).*

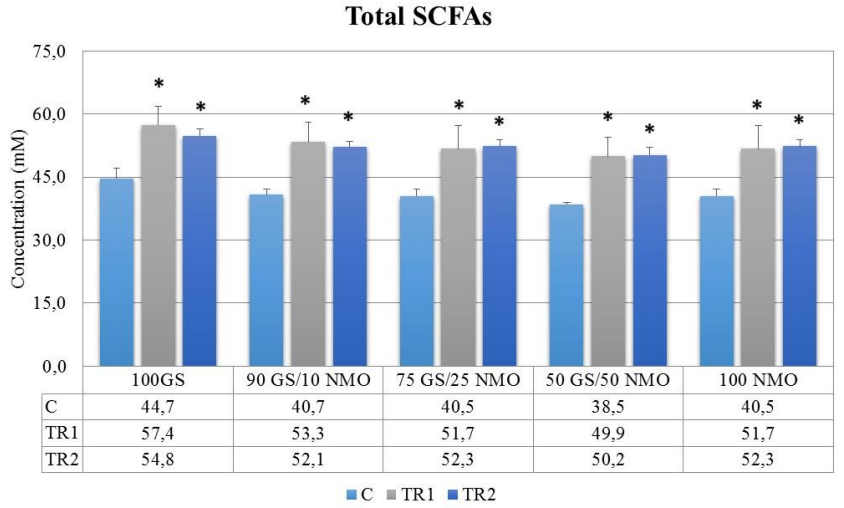
Butyrate levels (mM), displayed in Figure 30, demonstrated a more accentuated increase than propionate, although only during the 2nd treatment week (~2.0 mM increase vs control). In the 1st treatment week, there was a tendency to higher values, albeit not significantly different from the control period as this increase was gradual (except for 100GS).



	100GS	90 GS/10 NMO	75 GS/25 NMO	50 GS/50 NMO	100 NMO
C	8,8	8,9	8,6	9,5	8,6
TR1	10,8	10,8	10,6	12,1	10,6
TR2	11,5	11,5	11,0	11,2	11,0

Figure 30 - Butyrate levels (mM) during the control and treatment period (100/0, 90/10, 75/25, 50/50 and 0/100 of GS/NMO), in the proximal colon (PC) of the baby M-SHIME, inoculated with faecal material of baby 10 (n = 3).
** : difference against control statistically significant (p-value < 0.05).*

Altogether, **total SCFAs** (mM) levels, in Figure 31, increased during treatment weeks (~12mM increase vs control).



	100GS	90 GS/10 NMO	75 GS/25 NMO	50 GS/50 NMO	100 NMO
C	44,7	40,7	40,5	38,5	40,5
TR1	57,4	53,3	51,7	49,9	51,7
TR2	54,8	52,1	52,3	50,2	52,3

Figure 31 - Total SCFA levels (mM) during the control and treatment period (100/0, 90/10, 75/25, 50/50 and 0/100 of), in the proximal colon (PC) of the baby M-SHIME, inoculated with faecal material of baby 10 (n = 3).
** : difference against control statistically significant (p-value < 0.05).*

Lactate

Lactate analysis is of great interest due to his potential beneficial effects, specifically its antimicrobial effect or conversion to butyrate.

The results of this analysis are exposed in Figure 32 which shows the lactate concentration values (mM) of every PC (100/0, 90/10, 75/25, 50/50 and 0/100 of GS/NMO) during control (C1, 2 and 3) and treatment weeks (T1, 2, 3, 4, 5 and 6).

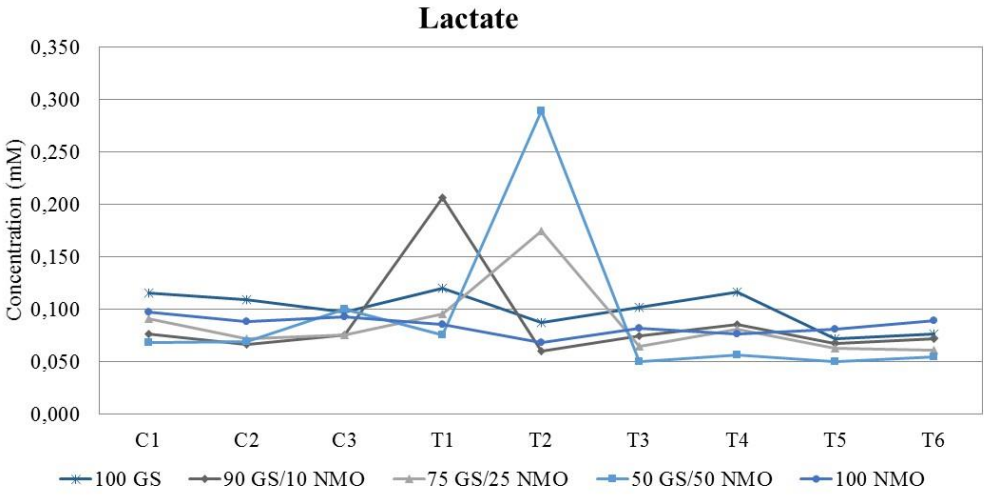


Figure 32 - Lactate levels (mM) during the control and treatment period (100/0, 90/10, 75/25, 50/50 and 0/100 of GS/NMO), in the proximal colon (PC) of the baby M-SHIME, inoculated with faecal material of baby 10 (n = 3).

During the two-week treatment period, all the PCs' profiles were low value (~1.0 mM) and very stable. However, there were several remarkable peaks upon 90GS/10NMO, 75GS/25NMO and 50GS/50NMO treatments, with the first two reaching around 2.0 mM and the latter, around 3.0 mM. These peaks were observed around the same time-lapse, particularly at T1 or T2.

Ammonium and branched SCFAs

Figure 33 shows the b-SCFA concentration (mM) values of every PC (100/0, 90/10, 75/25, 50/50 and 0/100 of GS/NMO) during control (C1, 2 and 3) and treatment weeks (T1, 2, 3, 4, 5 and 6). While there were no significant differences between the control and treatment weeks, similar as for lactate, a peak in the production of b-SCFAs was observed during the 1st treatment week, at the same time point (T3). The height of the peak increased for administration of (almost) pure products (100GS, 100NMO, 90GS/10NMO). When more similar ratios of GS/NMO were prepared, like 75/25 and especially 50/50, the peak in b-SCFAs levels became considerably lower.

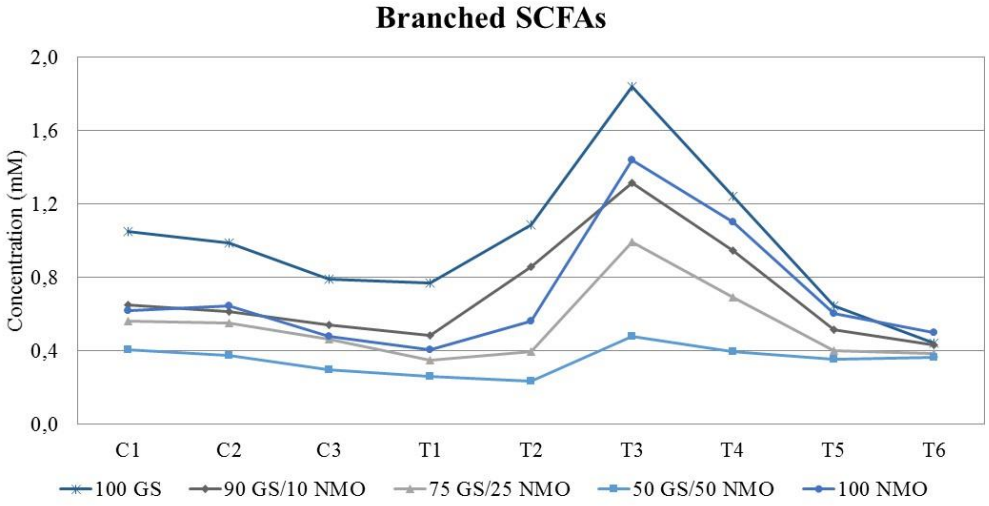


Figure 34 - Branched SCFA levels (mM) during the control and treatment period (100/0, 90/10, 75/25, 50/50 and 0/100 of GS/NMO), in the proximal colon (PC) of the baby M-SHIME, inoculated with faecal material of baby 10 (n = 3).

Regarding the NH_4^+ production, Figure 34 presents its concentration (mg NH_4^+-N/L) values on every PC (100/0, 90/10, 75/25, 50/50 and 0/100 of GS/NMO) during control (C1, 2 and 3) and treatment weeks (T1, 2, 3, 4, 5 and 6). This graph shows these values fairly stable over time, being kept between 150 and 200 mg (almost) continuously. Nonetheless, throughout the experiment there were some minor changes, with a significant decrease being noted for the 50 GS/50 NMO mixture, at T2, as we can see in Figure 35 which displays the average amount of ammonium (mg NH_4^+-N/L) per week.

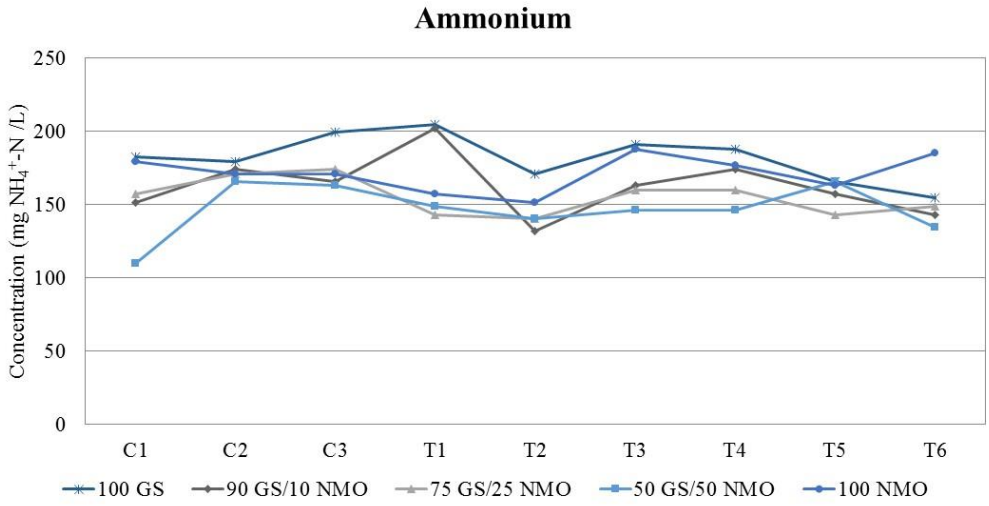
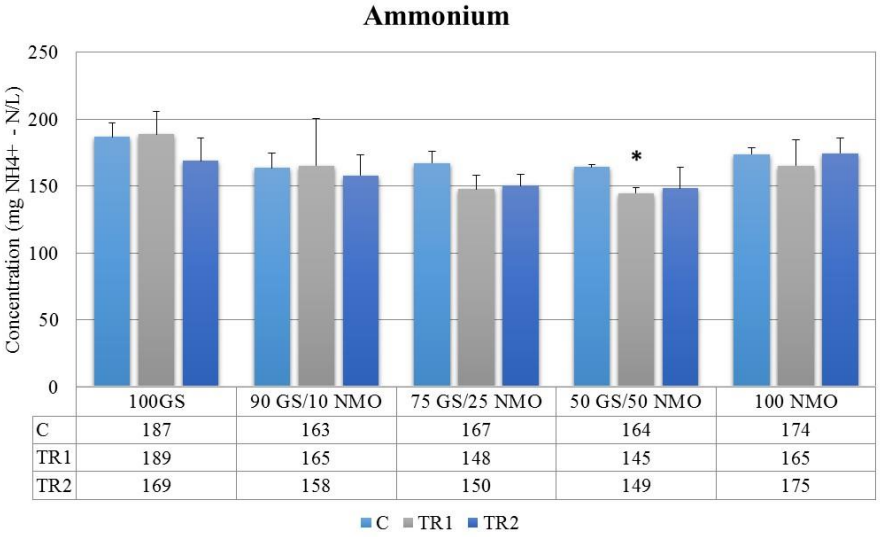


Figure 33 - Ammonium (mg NH_4^+-N/L) during the control and treatment period (100/0, 90/10, 75/25, 50/50 and 0/100 of GS/NMO), in the proximal colon (PC) of the baby M-SHIME, inoculated with faecal material of baby 10 (n per week = 3).



	100GS	90 GS/10 NMO	75 GS/25 NMO	50 GS/50 NMO	100 NMO
C	187	163	167	164	174
TR1	189	165	148	145	165
TR2	169	158	150	149	175

Figure 35 - Ammonium (mg NH_4^+-N/L) during the control and treatment period (100/0, 90/10, 75/25, 50/50 and 0/100 of GS/NMO), in the proximal colon (PC) of the baby M-SHIME, inoculated with faecal material of baby 10 (n per week = 3).
** : difference against control statistically significant (p-value < 0.05).*

Qualitative analysis of the Bifidobacterium *community composition (DGGE)*

The bifidobacterial community was analysed qualitatively. Figures 37 and 39 show the DGGE pictures upon 100/0, 90/10, 75/25, 50/50 and 0/100 of GS/NMO feeding conditions with respect to lumen and mucus sampling, respectively. Both pictures confirm, in concordance with the previous pre-screening experiment, that while GS stimulates *B. longum*, NMO increases the abundance of *B. longum* subsp. *infantis*. The selective increase of a specific *Bifidobacterium* species by GS and NMO became much clearer upon exporting the densities of each OTU from the DGGE profiles. These luminal and mucosal bifidobacterial abundances (based on the assumption that bands at the same height as during the previous project (2014) correspond with the same OTUs) are exposed in Figures 38 and 40.

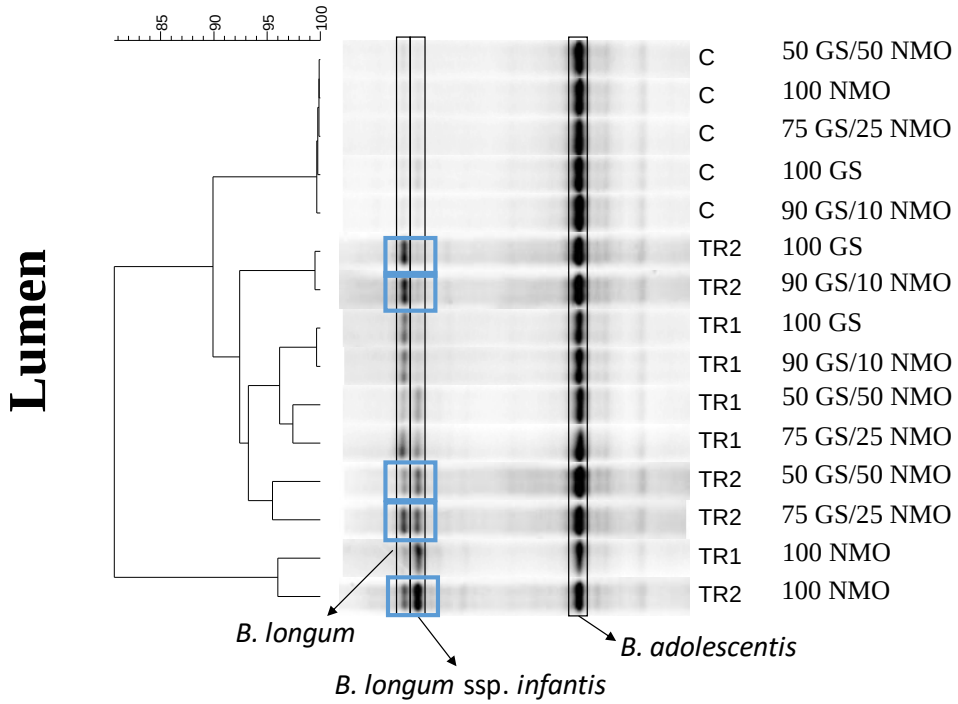


Figure 37 - Pearsson correlation of the bifidobacterial DGGE profiles of the luminal microbiota during the control and treatment period (100/0, 90/10, 75/25, 50/50 and 0/100 of GS/NMO), in the proximal colon (PC) of the baby M-SHIME, inoculated with faecal material of baby 10 (n per week = 1). The highlighted lanes concern 2nd treatment week. The reported phylogeny is based on the assumption that bands at the same height as during the previous project (2014) correspond with the same OTUs.

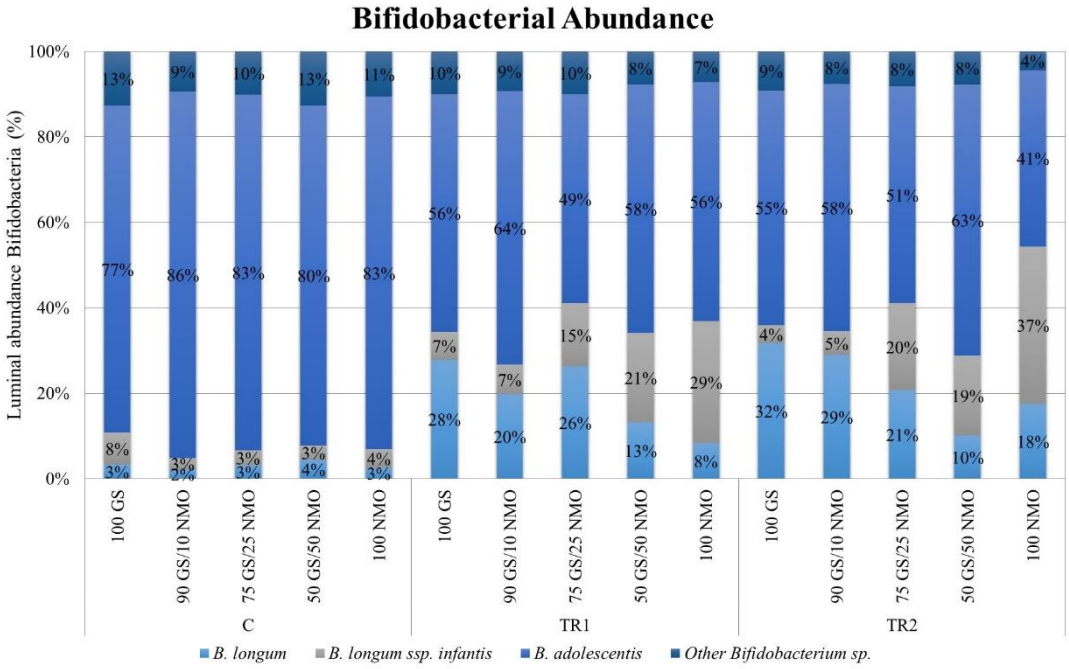


Figure 36 - Abundance of four Bifidobacterium species/groups based on the bifidobacterial DGGE profiles of the luminal microbiota during the control and treatment periods (100/0, 90/10, 75/25, 50/50 and 0/100 of GS/NMO), in the proximal colon (PC) of the baby M-SHIME, inoculated with faecal material of baby 10 (n per week = 1). The reported phylogeny is based on the assumption that bands at the same height as during the previous project (2014) correspond with the same OTUs.

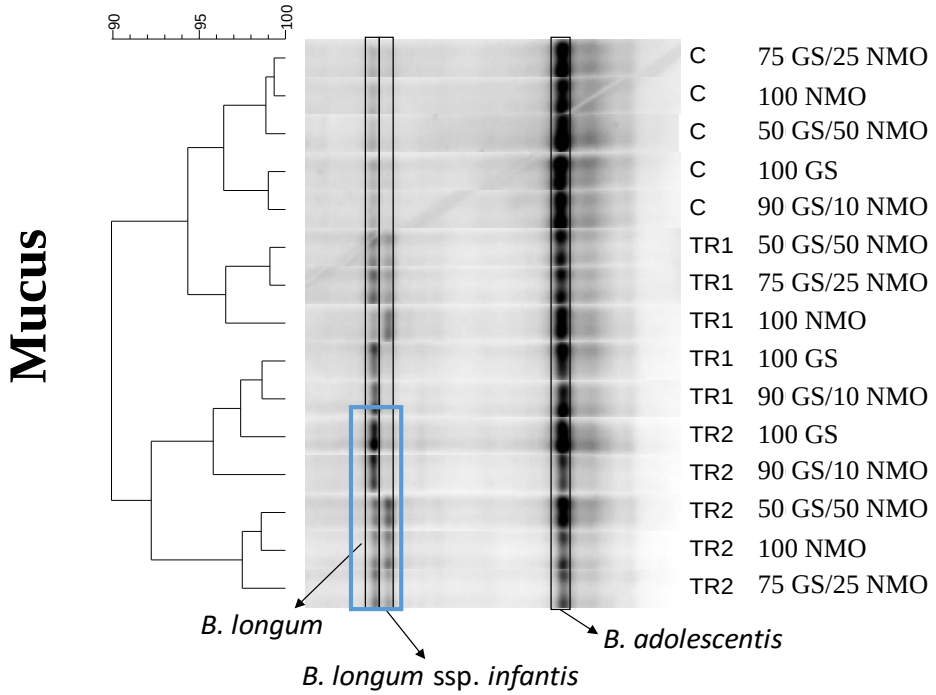


Figure 39 - Pearsson correlation of the bifidobacterial DGGE profiles of the mucosal microbiota during the control and treatment period (100/0, 90/10, 75/25, 50/50 and 0/100 of GS/NMO), in the proximal colon (PC) of the baby M-SHIME, inoculated with faecal material of baby 10 (n per week = 1). The highlighted lanes concern 2nd treatment week. The reported phylogeny is based on the assumption that bands at the same height as during the previous project (2014) correspond with the same OTUs.

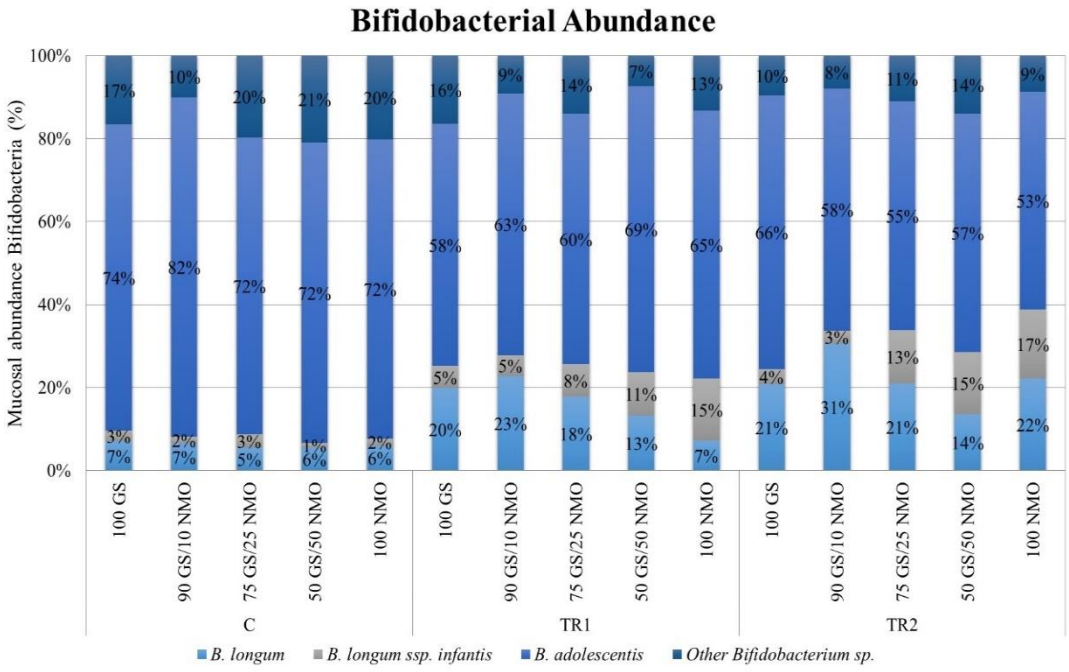


Figure 38 - Abundance of four Bifidobacterium species/groups based on the bifidobacterial DGGE profiles of the mucosal microbiota during the control and treatment periods (100/0, 90/10, 75/25, 50/50 and 0/100 of GS/NMO), in the proximal colon (PC) of the baby M-SHIME, inoculated with faecal material of baby 10 (n per week = 1). The reported phylogeny is based on the assumption that bands at the same height as during the previous project (2014) correspond with the same OTUs.

Quantitative analysis of the Bifidobacterium *community composition (qPCR)*

Specific qPCRs were selected, all together covering the main phyla/groups present in a baby gut microbiota, specifically: *Firmicutes*, *Lactobacilli*, *Bifidobacterium, Bacteroidetes* and *Enterobacterieceae* for luminal communities and the first three for mucosal communities.

A representative overview of the baby's community was obtained. The luminal results are displayed in the pie charts of Figure 40, each chart corresponding to a specific combination of GS/NMO at a different week of the experiment (C, TR1 or TR2).

One of the most remarkable features from these results is the fact that, during the 1st treatment week, all treatments tended to increase the *Firmicutes* and Bifidobacteria at the expense of *Enterobacterieceae*. Moreover, during the 2nd treatment week, NMO seemed to expand *Enterobacterieceae* levels in a dose-related way.

On the other hand, Figure 41 presents the graphs for the average mucosal abundance of *Firmicutes,* Bifidobacteria and *Lactobacilli* on every PC (100/0, 90/10, 75/25, 50/50 and 0/100 of GS/NMO) during control and treatment periods. The three groups were found to significantly expand at the intestinal gut wall, preferentially upon dosing higher levels of NMO as compared to GS.

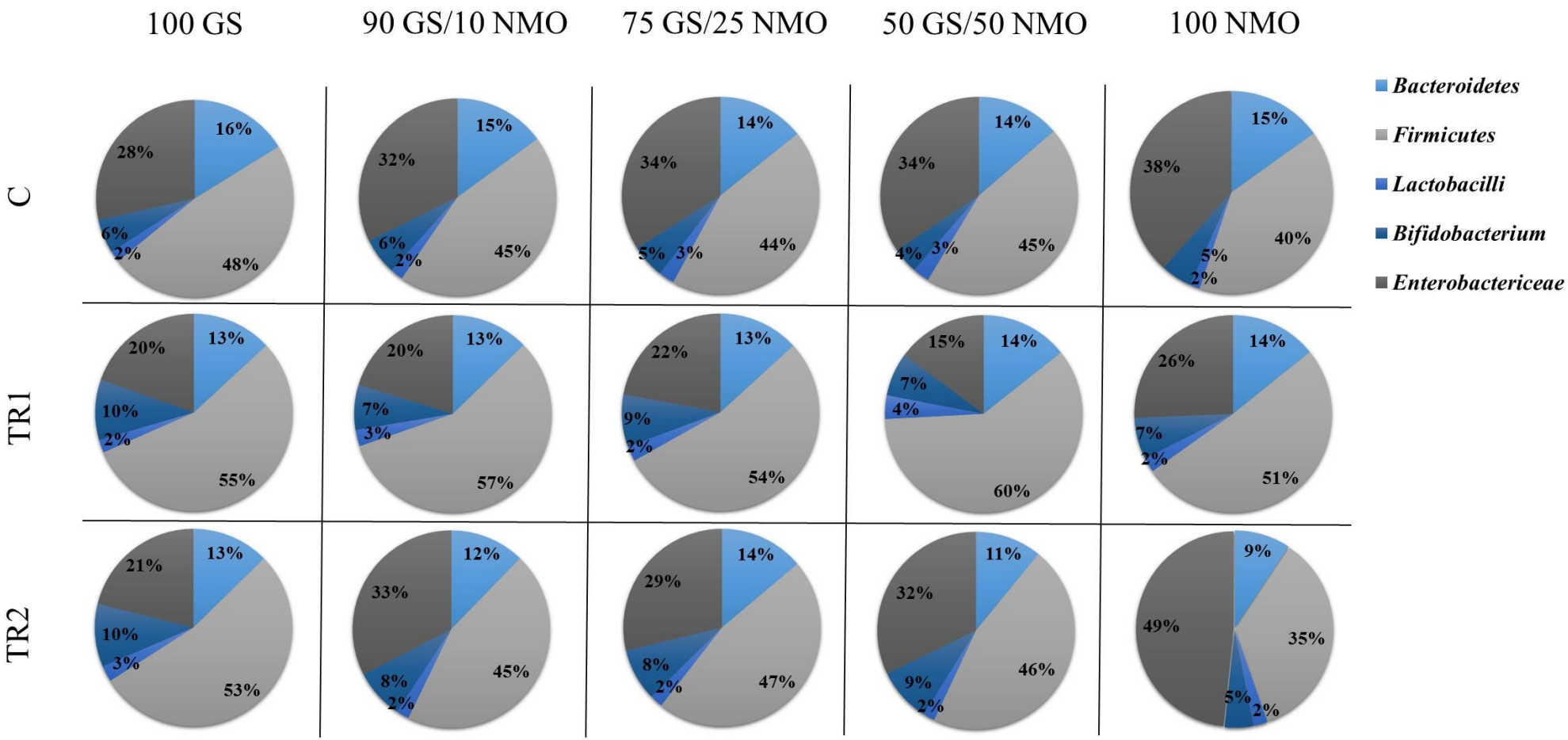
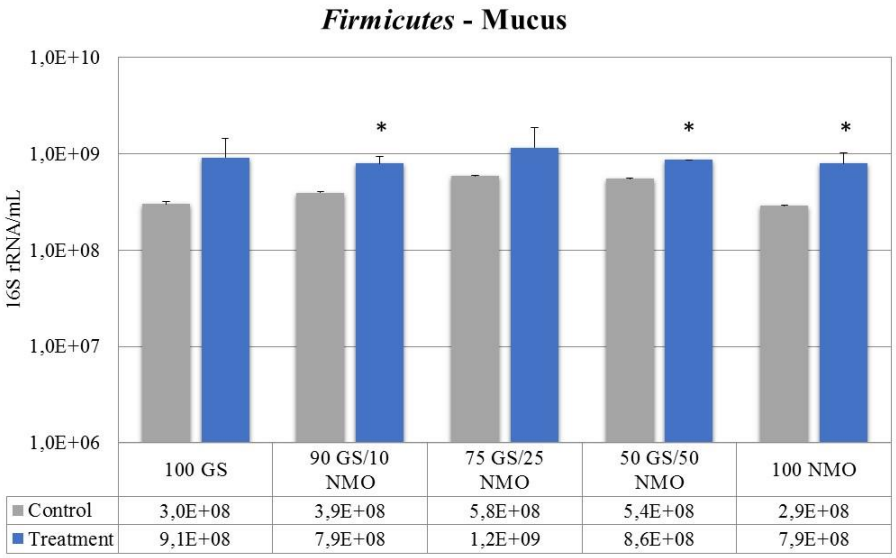
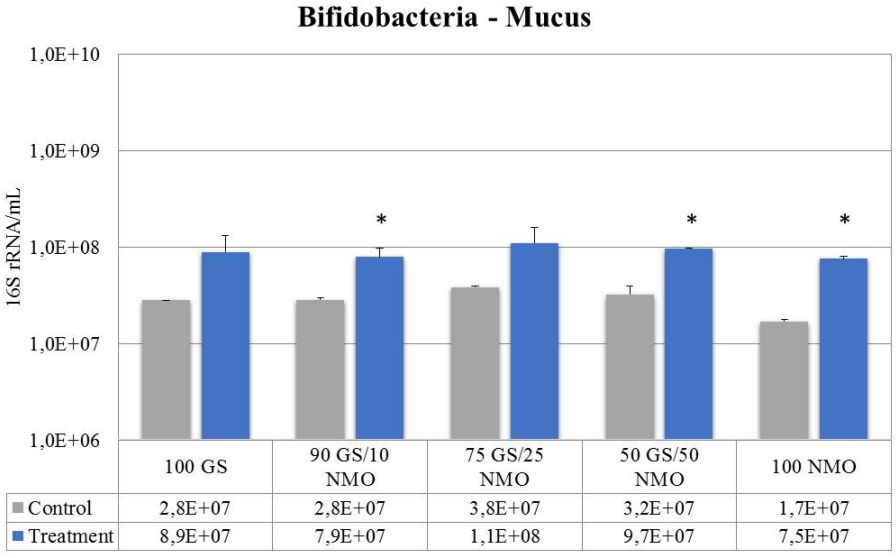


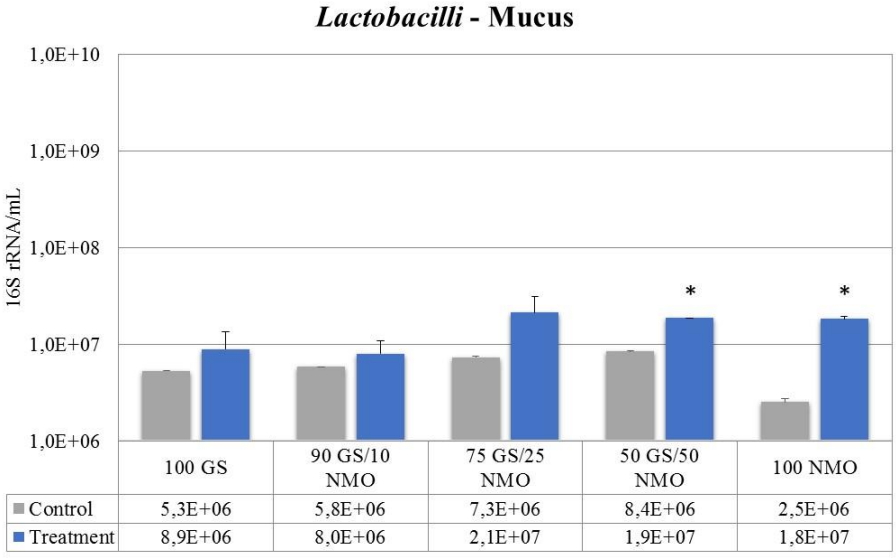
Figure 40 - Abundance of *Bacteroidetes*, *Firmicutes*, *Lactobacilli*, Bifidobacteria and Enterobacteria (%) in the luminal microbiota during the control and treatment period (100/0, 90/10, 75/25, 50/50 and 0/100 of GS/NMO), in the proximal colon (PC) of the baby M-SHIME, inoculated with faecal material of donor 10, as assessed with qPCR (n per week = 1).



	100 GS	90 GS/10 NMO	75 GS/25 NMO	50 GS/50 NMO	100 NMO
Control	3,0E+08	3,9E+08	5,8E+08	5,4E+08	2,9E+08
Treatment	9,1E+08	7,9E+08	1,2E+09	8,6E+08	7,9E+08



	100 GS	90 GS/10 NMO	75 GS/25 NMO	50 GS/50 NMO	100 NMO
Control	2,8E+07	2,8E+07	3,8E+07	3,2E+07	1,7E+07
Treatment	8,9E+07	7,9E+07	1,1E+08	9,7E+07	7,5E+07



	100 GS	90 GS/10 NMO	75 GS/25 NMO	50 GS/50 NMO	100 NMO
Control	5,3E+06	5,8E+06	7,3E+06	8,4E+06	2,5E+06
Treatment	8,9E+06	8,0E+06	2,1E+07	1,9E+07	1,8E+07

Figure 41 - Abundance of *Firmicutes*, *Lactobacilli* and Bifidobacteria (16S rRNA gene copies/mL) in the mucosal microbiota during the control and treatment period (100/0, 90/10, 75/25, 50/50 and 0/100 of GS/NMO), in the proximal colon (PC) of the baby M-SHIME, inoculated with faecal material of baby 10, as assessed with qPCR (n per week = 1). ** : difference against control statistically significant (p-value < 0.05).*

2. Discussion

Base-Acid consumption

The consumption of acid and base reflects the overall microbial activity throughout the SHIME experiment. Upon stabilization of the microbial community in the different reactors (starting from 2 weeks after inoculation), base-acid consumption is normally low. During treatment, bacteria may produce increased amounts of SCFAs and, as a consequence, the environment in the reactors will acidify, resulting in additional pH control by means of more administration of base to the respective reactors. As a result, the base-acid consumption will increase. By measuring the base-acid consumption throughout the experiment, the potential fermentation of GS, NMO and combinations thereof was estimated.

Analysing Figure 25 we may conclude that all five treatments were well fermented as they resulted in an immediate increase of base-acid consumption at the start of the treatment. When calculating the average values for the control and two treatment weeks (Figure 26), it followed that the increases during the 1st treatment week as compared to the control period were similar among the different treatments. Further, the values were also stable between the 1st and 2nd treatment weeks. This acidification is likely attributed to increased levels of SCFA and/or lactate production.

Despite the supplementation of only 3.2 g/L (~1.26 g/day), the increases in base versus acid consumption thus confirms that GS and NMO are well fermented, with metabolic changes in the PCs.

SCFAs

As a first remark, as it should, during the control period the SCFAs levels were very stable within (on average 95.6% similar between consecutive time points in control period) and reproducible between each of the 5 SHIME units (on average 93.9% similar between the five different units).

Secondly, the production of butyrate by the microbiota was the first sign for the presence of Bifidobacteria due to the process of *cross-feeding* (as explained in previous chapters).

Finally, these graphs reveal that, despite the relatively low dose of supplementation (3.2 g/L), GS, NMO and combinations thereof were strongly fermented resulting in enhanced SCFA levels. These increases are relevant since prebiotic properties

of a product may be evaluated by the increase of propionate and/or butyrate. Further, acetate can exert antimicrobial effects against pathogens and is therefore also considered as a beneficial microbial metabolite.

In conclusion, we may conclude that, because GS, NMO and combinations thereof increased the levels of all health-related SCFA (especially acetate, butyrate and only to minor extent propionate) in the proximal colons, they might thus result in distinct health benefits.

Lactate

As referred in the literature review, the human intestine harbours both lactate-producing and lactate-utilizing bacteria. When produced by lactic acid bacteria, lactate decreases the pH of the environment. Especially at low pH values, lactate can exert strong antimicrobial effects against pathogens as it becomes protonated. This protonated lactic acid can penetrate microbial cells after which it dissociates and releases protons within the cell, resulting in acidification and microbial cell death. Another beneficial effect of lactate results from its conversion to butyrate by specific lactate-utilizing butyrate-producing microorganisms such as *Anaerostipes caccae*, *Anaerostipes hadrus* or *Eubacterium hallii*. All this justifies the analysis of this metabolite.

Relatively to Figure 32, the peaks in lactate production depended on the exact GS/NMO mixture that was supplemented:

- Peak on day 1 after treatment for 90 GS/10 NMO (T1)
- Peak on day 3 after the start of the treatment with 75 GS/25 NMO and 50 GS/50 NMO (T2)
- No peak for 100 GS and 100 NMO. As a remark, there might also have been peaks in lactate production for 100 GS and 100 NMO but they might have occurred in between two sampling points.

As different microbial species thus produce and convert lactate, an increase of lactate concentration can both be the result of an increased production as well as a decreased conversion.

Interestingly, the time point at which the peak in lactate again decreased to normal levels (T2 for 90/10 and T3 for 75/25 and 50/50), corresponded with increased levels of butyrate (Figure 27). This reveals the *cross-feeding* of lactate to butyrate by *Clostridium* cluster IV or XIVa species. The fact that a peak in lactate levels appeared reveals that upon GS/NMO administration, the lactate production likely increased (by e.g.

Lactobacilli or Bifidobacteria), while it took several days for the butyrate producing species to adapt to these increased lactate levels in order to enhance the lactate consumption.

If that's the case, this increased conversion of lactate to butyrate would be particularly interesting given the anti-inflammatory and anti-carcinogenic effects that are attributed to butyrate.

Ammonium and branched SCFAs

Both the production of ammonium (NH_4^+) and branched SCFAs (b-SCFA = sum of isobutyrate, isovalerate and isocaproate) are the result of protein degradation and reflective for the proteolytic activity of the gut microbiota. As the latter has been associated with direct and indirect detrimental health effects (e.g. colon carcinogenesis), a reduction in ammonium/b-SCFA production is considered as beneficial.

Despite the very low dose of supplementation (3.2 g/L ~1.26g/day), the 50GS/50NMO mixture thus significantly decreased the levels of NH_4^+, while also not resulting in a peak of b-SCFA.

Qualitative analysis of the Bifidobacterium community composition (DGGE)

The first observation concerning both luminal and mucosal DGGE pictures, relates do the control lanes (Figures 37 and 39). It is clear that the same enhanced band, referring to *B. adolescentis*, appears for every GS/NMO combination proving that, during control, every PC held the same bifidobacterial community and posterior changes were due to the different treatments applied.

Secondly, as mentioned beforehand, when examining the DGGE pictures, two major conclusions can be withdrew: that GS stimulates *B. longum* and NMO increases the abundance of *B. longum* subsp. *infantis*. Further, there was a clear dose-response effect of increasing levels of NMO or GS on stimulation of the *Bifidobacterium* species that they selectively target.

Looking closely at the DGGE picture related to lumen (Figure 37), more particularly to 2nd treatment week (TR2) lanes (highlighted with blue squares), there is a gradual decrease of the intensity of the *B. longum* band. Simultaneously, the GS percentage decreases. This means that the abundance of *B. longum* is higher with 100GS (darkest band) than with 100NMO (lightest band).

In case of *B. longum* subsp. i*nfantis*, the darkest band showed up with 100NMO, while the lightest appeared with 100GS. For the two bacteria, this gradual increase/decrease in band intensity was also obvious even for the intermediate combinations of the test products, demonstrating a high degree of sensitivity of the bifidobacterial community to NMO (and GS).

This abundance variance was alternatively exposed in a proportion graph (Figure 38). Upon 100NMO, *B. longum* subsp. *infantis* increases drastically from 4% (control week) to 29%, and again to 37% (1st and 2nd treatment weeks, respectively). As well as the lumen, the mucus also showed the same pattern of variance for *B. longum* and *B. longum* subsp. *infantis*. The exact abundances of the latter for 100NMO were 2%, 15% and 17% at control and both treatment weeks, respectively. Once again, an accentuated increase of this specific *Bifidobacterium* is observable. This stimulation of specific Bifidobacteria promotes their growth over *B. adolescentis* and other *Bifidobacterium* sp..

In order to focus on the NMO-mediated increase of *B. longum* subsp. i*nfantis*, additional graphs focussing on this species were constructed and are presented in Figures 41 and 42. It followed that from 25% NMO on, a decent stimulation of this species is observed upon a 2-week treatment period.

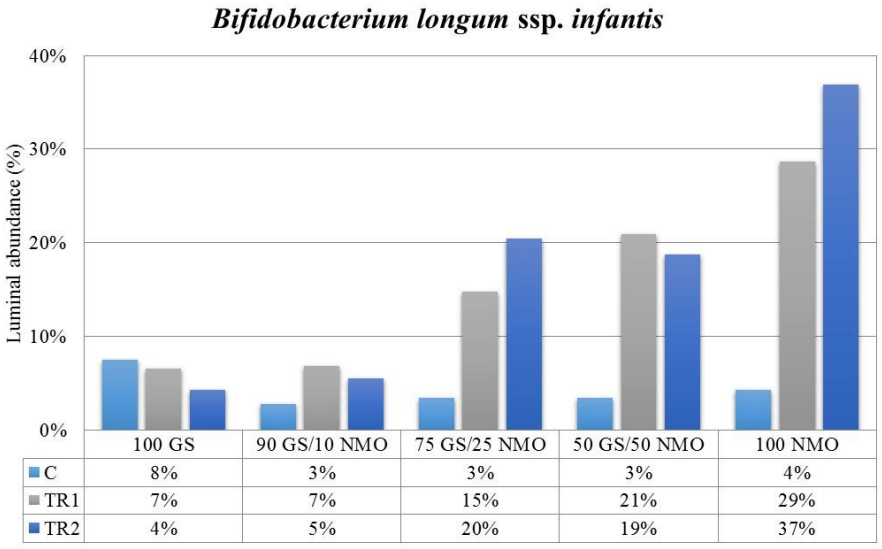
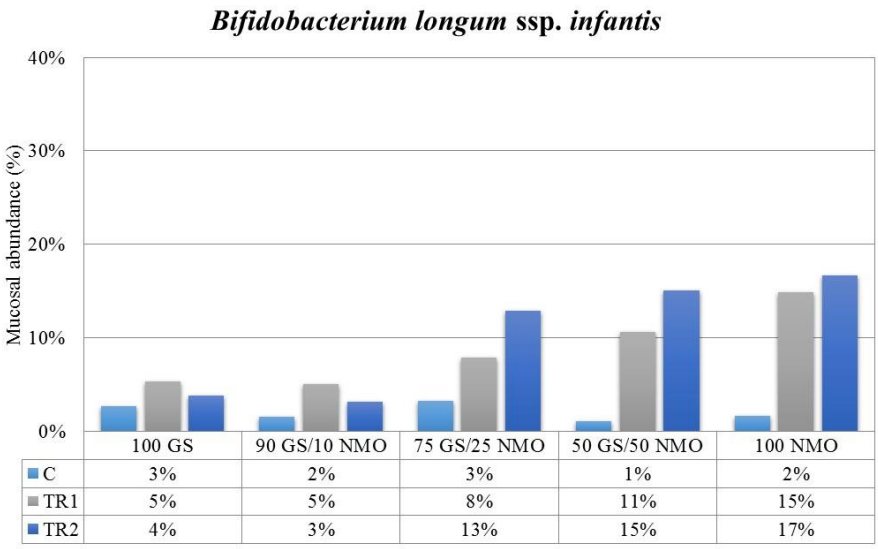


Figure 42 - Abundance of *B. longum* subsp. *infantis* in the bifidobacterial DGGE profiles of the luminal microbiota during the control and treatment period (100/0, 90/10, 75/25, 50/50 and 0/100 of GS/NMO), in the proximal colon (PC) of the baby M-SHIME, inoculated with faecal material of baby 10 (n per week = 1). The phylogeny is based on the assumption that bands at the same height as during the previous project (2014) correspond with the same OTUs.



	100 GS	90 GS/10 NMO	75 GS/25 NMO	50 GS/50 NMO	100 NMO
C	3%	2%	3%	1%	2%
TR1	5%	5%	8%	11%	15%
TR2	4%	3%	13%	15%	17%

Figure 43 - Abundance of *B. longum* subsp. *infantis* in the bifidobacterial DGGE profiles of the mucosal microbiota during the control and treatment period (100/0, 90/10, 75/25, 50/50 and 0/100 of GS/NMO), in the proximal colon (PC) of the baby M-SHIME, inoculated with faecal material of baby 10 (n per week = 1). The phylogeny is based on the assumption that bands at the same height as during the previous project (2014) correspond with the same OTUs.

Quantitative analysis of the Bifidobacterium *community composition (qPCR)*

Relevant ratios of different microbial groups were obtained during the control period, as compared to *in vivo* data. As an example, the graph below published by Fan et al. (2013), reports a microbiota composition of breast-fed infants at phylum level with levels of around 62 % *Firmicutes*, 21% Proteobacteria, 7% Actinobacteria and 10% others (including *Bacteroidetes*). Average levels obtained during the control period included 44 (± 3%), 33 (± 4%), 5 (± 2%) and 17 (± 1%), respectively.

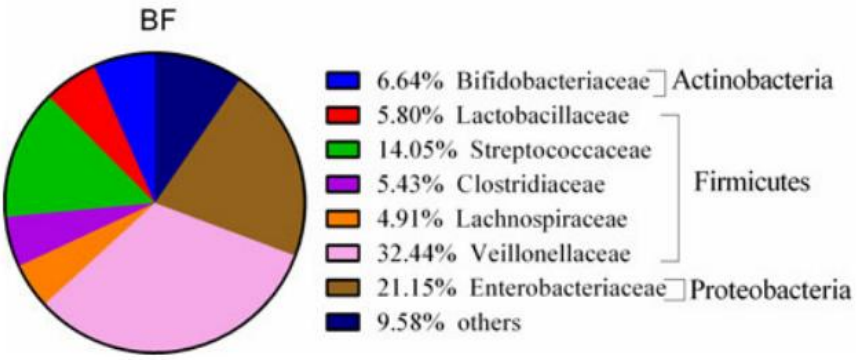


Figure 44 - Microbiota composition of breast-fed infants at phylum level (Fan et al., 2013).

During the 1st treatment week, all treatments tended to increase the *Firmicutes* (diverse phylum including many butyrate producers) and Bifidobacteria (containing health-related species) at the expense of Enterobacteria (containing disease-related microbes).

While above mentioned beneficial community shifts were stable for GS during the 2nd treatment week, NMO seemed to expand Enterobacteria levels in a dose-related way. This finding probably relates to recent studies showing that HMOs tend to increase Proteobacteria as this phylum contains members that are capable of *cross-feeding* on HMOs (Charbonneau et al., 2016; Frese et al., 2015). This means that these species are not necessarily primary degraders of NMO but rather benefit from degradation products of NMO that are produced by other microbes (like probably *B. longum* ssp. *infantis*). Given the fact that Enterobacteria also contain opportunistic pathogens, dosing 100% NMO might increase the risk of an intervention in vulnerable babies. On the other hand, Enterobacteria have also been shown to be essential for a correct priming of the immune system (Mai et al., 2011).

VI. CONCLUSION

This study was focused on the response scenarios in terms of prebiotic effects, mainly on the assessment of the bifidogenic effects, of different mixtures of a conventional prebiotic ('golden standard') and a novel HMO in an infant's microbiota.

The first part of the project, a pre-screening, was performed in order to select the fittest faecal donor as this experiment provided valuable information on the inter-individual differences among babies in response to NMO administration. While it is well known that the early-life microbiota composition can differ a lot among babies, it was interesting to observe that this also resulted in different response scenarios to NMO administration.

Overall, 7 out of the 10 donors responded strongly to the NMO treatment resulting in an acidification of the medium as well as a bifidogenic effect, despite the fact that the NMO breakdown was likely mediated by different *Bifidobacterium* sp. in each of the different donors. NMO degradation can thus occur via several different ways, which is a pre-requisite for a good prebiotic as the infant gut microbiota is highly variable in terms of microbial colonization.

Knowing that there are several response scenarios, it was decided to focus on the mechanism of action that was identified during a previous project and that was confirmed by the results of donor 10, in which NMO degradation was mediated by a specific species, specifically, *B. longum* subsp. *infantis*.

The second part of the project was consisted on a baby M-SHIME, used to simulate the conditions of the GIT of babies, as described by De Boever et al. (2001). For this part of the study, the faecal sample of a single donor (donor 10) was used making this project still exploratory.

As it should, during the control period, base-acid consumption, SCFAs, lactate, ammonium and microbiota composition were all very stable within and reproducible between each of the 5 PCs. This provided an excellent platform to benchmark the different mixtures of GS and NMO.

Despite the supplementation of only 3.2 g/L (~ 1.26 g/day), the increases in base versus acid consumption confirmed that GS, NMO and combinations thereof were well fermented, with metabolic changes in the PCs. These changes included increased levels of all health-related SCFAs (especially acetate, butyrate and only to minor extent propionate). Shortly after the start of the treatment, a peak in lactate levels was observed,

likely due to enhanced production upon GS/NMO administration (by e.g. *Lactobacilli* or Bifidobacteria). The subsequent decrease in lactate levels corresponded with increased butyrate levels. This observation can be attributed to *cross-feeding* of lactate to butyrate by *Clostridium* cluster IV or XIVa species, which is particularly interesting given the anti-inflammatory and anticarcinogenic effects of butyrate. A final metabolic result was that only minor decreases were observed on proteolytic markers. The treatment with significantly decreased levels of NH_4^+ was the treatment with 50GS/50NMO.

Regarding overall community changes, it followed that mucosal levels of Bifidobacteria, *Lactobacilli* and *Firmicutes* (diverse phylum including many butyrate producers), increased significantly upon dosing higher levels of NMO as compared to GS. With respect to luminal levels, it was found that during the 1st treatment week, all treatments tended to beneficially modify the baby microbiota with increases of *Firmicutes* and Bifidobacteria at the expense of Enterobacteria (containing disease-related microbes). During the 2nd week, it followed that NMO increased Enterobacteria levels in a dose-related way. This finding probably relates to recent studies showing that HMOs tend to increase Proteobacteria as this phylum contains members (e.g. *E. coli* < Enterobacteria) that are capable of *cross-feeding* on HMOs. Given the fact that Enterobacteria also contain opportunistic pathogens, dosing would be recommended over 100NMO in order to avoid such a bloom in Enterobacteria.

In conclusion, the optimal NMO dose might be the dose at which there is still a strong stimulation of *B. longum* ssp. i*nfantis*, and health-related SCFA, yet also being a dose that does not result in a major expansion of Enterobacteria. That being so, 75GS/25NMO or 50GS/50NMO seem excellent candidates.

VII. BIBLIOGRAPHY

Aagaard, K., Ma, J., Antony, K.M., Ganu, R., Petrosino, J., Versalovic, J., 2014. The Placenta Harbors a Unique Microbiome. Sci. Transl. Med. 6, 1–11.

Adlerberth, I., Wold, A., 2009. Establishment of the gut microbiota in Western infants. Acta Paediatr. 98, 229–38.

Alander, M., De Smet, I., Nollet, L., Verstraete, W., von Wright, A., Mattila-Sandholm, T., 1999. The effect of probiotic strains on the microbiota of the Simulator of the Human Intestinal Microbial Ecosystem (SHIME). Int. J. Food Microbiol. 46, 71–79.

Angelakis, E., Merhej, V., Raoult, D., 2013. Related actions of probiotics and antibiotics on gut microbiota and weight modification. Lancet Infect. Dis. 13, 889–99.

Aura, A.-M., Oikarinen, S., Mutanen, M., Heinonen, S.-M., Adlercreutz, H.C.T., Virtanen, H., Poutanen, K.S., 2006. Suitability of a batch in vitro fermentation model using human faecal microbiota for prediction of conversion of flaxseed lignans to enterolactone with reference to an in vivo rat model. Eur. J. Nutr. 45, 45–51.

Ben, X.M., Li, J., Feng, Z.T., Shi, S.Y., Lu, Y.D., Chen, R., Zhou, X.Y., 2008. Low level of galacto-oligosaccharide in infant formula stimulates growth of intestinal Bifidobacteria and Lactobacilli. World J. Gastroenterol. 14, 6564–68.

Bezirtzoglou, E., 1997. The Intestinal Microflora During the First Weeks of Life. Anaerobe 3, 173–77.

Bezirtzoglou, E., Stavropoulou, E., 2011. Immunology and probiotic impact of the newborn and young children intestinal microflora. Anaerobe 17, 369–74.

Bezirtzoglou, E., Tsiotsias, A., Welling, G.W., 2011. Microbiota profile in feces of breast- and formula-fed newborns by using fluorescence in situ hybridization (FISH). Anaerobe 17, 478–82.

Bianchi, F., Rossi, E.A., Sakamoto, I.K., Adorno, M.A.T., Van de Wiele, T., Sivieri, K., 2014. Beneficial effects of fermented vegetal beverages on human gastrointestinal microbial ecosystem in a simulator. Food Res. Int. 64, 43–52.

Braegger, C., Chmielewska, A., Decsi, T., Kolacek, S., Mihatsch, W., Moreno, L., Pieścik, M., Puntis, J., Shamir, R., Szajewska, H., Turck, D., van Goudoever, J., 2011. Supplementation of Infant Formula With Probiotics and/or Prebiotics: A Systematic Review and Comment by the ESPGHAN Committee on Nutrition. J. Pediatr. Gastroenterol. Nutr. 52, 238–50.

Cammarota, G., Ianiro, G., Bibbò, S., Gasbarrini, A., 2014. Gut microbiota modulation: probiotics, antibiotics or fecal microbiota transplantation? Intern. Emerg. Med. 9, 365–73.

Cammarota, G., Ianiro, G., Cianci, R., Bibbò, S., Gasbarrini, A., Currò, D., 2015. The involvement of gut microbiota in inflammatory bowel disease pathogenesis: potential for therapy. Pharmacol. Ther. 149, 191–212.

Cani, P.D., Bibiloni, R., Knauf, C., Waget, A., Neyrinck, A.M., Delzenne, N.M., Burcelin, R., 2008. Changes in Gut Microbiota Control Metabolic Endotoxemia-Induced Inflammation in High-Fat Diet-Induced Obesity and Diabetes in Mice. Diabetes 57, 1470–81.

Cénit, M.C., Matzaraki, V., Tigchelaar, E.F., Zhernakova, A., 2014. Rapidly expanding knowledge on the role of the gut microbiome in health and disease. Biochim. Biophys. Acta - Mol. Basis Dis. 1842, 1981–92.

Charbonneau, M.R., O'Donnell, D., Blanton, L.V., Totten, S.M., Davis, J.C.C., Barratt, M.J., Cheng, J., Guruge, J., Talcott, M., Bain, J.R., Muehlbauer, M.J., Ilkayeva, O., Wu, C., Struckmeyer, T., Barile, D., Mangani, C., Jorgensen, J., Fan, Y., Maleta, K., Dewey, K.G., Ashorn, P., Newgard, C.B., Lebrilla, C., Mills, D.A., Gordon, J.I., 2016. Sialylated Milk Oligosaccharides Promote Microbiota-Dependent Growth in Models of Infant Undernutrition. Cell 164, 859–871.

Clarke, G., Stilling, R.M., Kennedy, P.J., Stanton, C., Cryan, J.F., Dinan, T.G., 2014. Minireview: Gut Microbiota: The Neglected Endocrine Organ. Mol. Endocrinol. 28, 1221–38.

Collado, M.C., Delgado, S., Maldonado, A., Rodríguez, J.M., 2009. Assessment of the bacterial diversity of breast milk of healthy women by quantitative real-time PCR. Lett. Appl. Microbiol. 48, 523–28.

Coppa, G. V, Gabrielli, O., Zampini, L., Galeazzi, T., Ficcadenti, A., Padella, L., Santoro, L., Soldi, S., Carlucci, A., Bertino, E., Morelli, L., 2011. Oligosaccharides in 4 Different Milk Groups, Bifidobacteria, and Ruminococcus obeum. J. Pediatr. Gastroenterol. Nutr. 53, 80–87.

De Boever, P., Wouters, R., Vermeirssen, V., Boon, N., Verstraete, W., 2001. Development of a Six-Stage Culture System for Simulating the Gastrointestinal Microbiota of Weaned Infants. Microb. Ecol. Health Dis. 13, 111–23.

De Filippo, C., Cavalieri, D., Di Paola, M., Ramazzotti, M., Poullet, J.B., Massart, S., Collini, S., Pieraccini, G., Lionetti, P., 2010. Impact of diet in shaping gut microbiota revealed by a comparative study in children from Europe and rural Africa. Proc. Natl. Acad. Sci. 107, 14691–96.

De Leoz, M.L. a, Kalanetra, K.M., Bokulich, N. a, Strum, J.S., Underwood, M. a, German, J.B., Mills, D. a, Lebrilla, C.B., 2015. Human Milk Glycomics and Gut Microbial Genomics in Infant Feces Show a Correlation between Human Milk Oligosaccharides and Gut Microbiota: A Proof-of-Concept Study. J. Proteome Res. 14, 491–502.

De Weirdt, R., Possemiers, S., Vermeulen, G., Moerdijk-Poortvliet, T.C.W., Boschker, H.T.S., Verstraete, W., Van de Wiele, T., 2010. Human faecal microbiota display variable patterns of glycerol metabolism. FEMS Microbiol. Ecol. 74, 601–11.

Dethlefsen, L., McFall-Ngai, M., Relman, D.A., 2007. An ecological and evolutionary perspective on human–microbe mutualism and disease. Nature 449, 811–18.

Dominguez-Bello, M.G., Costello, E.K., Contreras, M., Magris, M., Hidalgo, G., Fierer, N., Knight, R., 2010. Delivery mode shapes the acquisition and structure of the initial microbiota across multiple body habitats in newborns. Proc. Natl. Acad. Sci. 107, 11971–75.

Donovan, S., Gibson, G., Newburg, D., 2009. Prebiotics in Infant Nutrition.

Erwin G. Zoetendal, A.D.L.A., 2001. The Host Genotype Affects the Bacterial Community in the Human Gastronintestinal Tract. Microb. Ecol. Health Dis. 13, 129–34.

Fan, W., Huo, G., Li, X., Yang, L., Duan, C., Wang, T., Chen, J., 2013. Diversity of the intestinal microbiota in different patterns of feeding infants by Illumina high-throughput sequencing. World J. Microbiol. Biotechnol. 29, 2365–2372.

Fanaro, S., Chierici, R., Guerrini, P., Vigi, V., 2007. Intestinal microflora in early infancy: composition and development. Acta Paediatr. 441, 48–55.

Favier, C.F., de Vos, W.M., Akkermans, A.D.., 2003. Development of bacterial and bifidobacterial communities in feces of newborn babies. Anaerobe 9, 219–29.

Flint, H.J., Scott, K.P., Duncan, S.H., Louis, P., Forano, E., 2012. Microbial degradation of complex carbohydrates in the gut. Gut Microbes 3, 289–306.

Food and Agriculture Organization of the United Nations & World Health Organization, 2002. Guidelines for the Evaluation of Probiotics in Food, Report on Drafting Guidelines for the Evaluation of Probiotics in Food. London,Ontario,Canada.

Francino, M., 2014. Early Development of the Gut Microbiota and Immune Health. Pathogens 3, 769–90.

Frese, S.A., Parker, K., Calvert, C.C., Mills, D.A., 2015. Diet shapes the gut microbiome of pigs during nursing and weaning. Microbiome 3, 28.

Gritz, E.C., Bhandari, V., 2015. The Human Neonatal Gut Microbiome: A Brief Review. Front. Pediatr. 3, 1–12.

Hollister, E.B., Riehle, K., Luna, R.A., Weidler, E.M., Rubio-Gonzales, M., Mistretta, T.-A., Raza, S., Doddapaneni, H. V., Metcalf, G.A., Muzny, D.M., Gibbs, R.A., Petrosino, J.F., Shulman, R.J., Versalovic, J., 2015. Structure and function of the healthy pre-adolescent pediatric gut microbiome. Microbiome 3, 1–13.

Holscher, H.D., Faust, K.L., Czerkies, L. a., Litov, R., Ziegler, E.E., Lessin, H., Hatch, T., Sun, S., Tappenden, K. a., 2012. Effects of Prebiotic-Containing Infant Formula on Gastrointestinal Tolerance and Fecal Microbiota in a Randomized Controlled Trial. J. Parenter. Enter. Nutr. 36, 95–105.

Hoover, D.G., 2014. Bifidobacterium, in: Batt, C.A., Tortorello, M. Lou (Eds.), Encyclopedia of Food Microbiology. Elsevier, Newark, USA, pp. 216–22.

Kapiki, A., Costalos, C., Oikonomidou, C., Triantafyllidou, A., Loukatou, E., Pertrohilou, V., 2007. The effect of a fructo-oligosaccharide supplemented formula on gut flora of preterm infants. Early Hum. Dev. 83, 335–44.

Kavanaugh, D.W., O'Callaghan, J., Buttó, L.F., Slattery, H., Lane, J., Clyne, M., Kane, M., Joshi, L., Hickey, R.M., 2013. Exposure of Bifidobacterium longum subsp. infantis to Milk Oligosaccharides Increases Adhesion to Epithelial Cells and Induces a Substantial Transcriptional Response. PLoS One 8.

Kirmiz, N., Mills, D.A., 2016. Intestinal Microbiota in Breast-Fed Infants: Insights into Infant-Associated Bifidobacteria and Human Milk Glycans, in: Watson, R.R., Preedy, V.R. (Eds.), Probiotics, Prebiotics, and Synbiotics - Bioactive Foods in Health Promotion. Academic Press, pp. 59–73.

Koletzko, B., 2015. Formula Feeding, in: Koletzko, B. (Ed.), Pediatric Nutrition in Practice. Karger, Basel, pp. 97–103.

Kondepudi, K.K., Ambalam, P., Nilsson, I., Wadström, T., Ljungh, Å., 2012. Prebiotic-non-digestible oligosaccharides preference of probiotic bifidobacteria and antimicrobial activity against Clostridium difficile. Anaerobe 18, 489–97.

Leahy, S.C., Higgins, D.G., Fitzgerald, G.F., Sinderen, D., 2005. Getting better with bifidobacteria. J. Appl. Microbiol. 98, 1303–15.

Lopetuso, L.R., Scaldaferri, F., Petito, V., Gasbarrini, A., 2013. Commensal Clostridia: leading players in the maintenance of gut homeostasis. Gut Pathog. 5, 23–31.

Louis, P., Hold, G.L., Flint, H.J., 2014. The gut microbiota, bacterial metabolites and colorectal cancer. Nat. Rev. Microbiol. 12, 661–72.

Mai, V., Young, C.M., Ukhanova, M., Wang, X., Sun, Y., Casella, G., Theriaque, D., Li, N., Sharma, R., Hudak, M., Neu, J., 2011. Fecal microbiota in premature infants prior to

necrotizing enterocolitis. PLoS One 6, e20647.

Marques, T.M., Wall, R., Ross, R.P., Fitzgerald, G.F., Ryan, C.A., Stanton, C., 2010. Programming infant gut microbiota: influence of dietary and environmental factors. Curr. Opin. Biotechnol. 21, 149–56.

Martín, R., Miquel, S., Ulmer, J., Langella, P., Bermúdez-Humarán, L.G., 2014. Gut ecosystem: how microbes help us. Benef. Microbes 5, 219–33.

Matamoros, S., Gras-Leguen, C., Le Vacon, F., Potel, G., de La Cochetiere, M.-F., 2013. Development of intestinal microbiota in infants and its impact on health. Trends Microbiol. 21, 167–73.

Mischke, M., Plosch, T., 2013. More than just a gut instinct-the potential interplay between a baby's nutrition, its gut microbiome, and the epigenome. AJP Regul. Integr. Comp. Physiol. 304, 1065–69.

Molly, K., Woestyne, M. Vande, Smet, I. De, Verstraete, W., 1994. Validation of the Simulator of the Human Intestinal Microbial Ecosystem (SHIME) Reactor Using Microorganism-associated Activities. Microb. Ecol. Health Dis. 7, 191–200.

Moro, G., Minoli, I., Mosca, M., Fanaro, S., Jelinek, J., Stahl, B., Boehm, G., 2002. Dosage-related bifidogenic effects of galacto- and fructooligosaccharides in formula-fed term infants. J. Pediatr. Gastroenterol. Nutr. 34, 291–295.

Muñoz, J.A.M., Chenoll, E., Casinos, B., Bataller, E., Ramón, D., Genovés, S., Montava, R., Ribes, J.M., Buesa, J., Fàbrega, J., Rivero, M., 2011. Novel probiotic Bifidobacterium longum subsp. infantis CECT 7210 strain active against rotavirus infections. Appl. Environ. Microbiol. 77, 8775–8783.

Munyaka, P.M., Khafipour, E., Ghia, J.-E., 2014. External influence of early childhood establishment of gut microbiota and subsequent health implications. Front. Pediatr. 2, 1–9.

Nylund, L., Satokari, R., Salminen, S., de Vos, W.M., 2014. Intestinal microbiota during early life - impact on health and disease. Proc. Nutr. Soc. 73, 457–69.

O'Hara, A.M., Shanahan, F., 2006. The gut flora as a forgotten organ. EMBO Rep. 7, 688–93.

Park, H.-K., Shim, S.-S., Kim, S.-Y., Park, J.-H., Park, S.-E., Kim, H.-J., Kang, B.-C., Kim, C.-M., 2005. Molecular analysis of colonized bacteria in a human newborn infant gut. J. Microbiol. 43, 345–53.

Patel, S., Goyal, A., 2012. The current trends and future perspectives of prebiotics research: a review. 3 Biotech 2, 115–125.

Payne, A.N., Zihler, A., Chassard, C., Lacroix, C., 2012. Advances and perspectives in in vitro human gut fermentation modeling. Trends Biotechnol. 30, 17–25.

Penders, J., Thijs, C., Vink, C., Stelma, F.F., Snijders, B., Kummeling, I., van den Brandt, P.A., Stobberingh, E.E., 2006. Factors Influencing the Composition of the Intestinal Microbiota in Early Infancy. Pediatrics 118, 511–21.

Possemiers, S., Bolca, S., Grootaert, C., Heyerick, A., Decroos, K., Dhooge, W., De Keukeleire, D., Rabot, S., Verstraete, W., Van de Wiele, T., 2006. The prenylflavonoid isoxanthohumol from hops (Humulus lupulus L.) is activated into the potent phytoestrogen 8-prenylnaringenin in vitro and in the human intestine. J. Nutr. 136, 1862–67.

ProDigest.eu - Simulator of Human Intestinal Microbial Ecosystem (SHIME®) [WWW Document], n.d. URL http://www.prodigest.eu/EN/technology/2simulatorofhumanintestinalmicrobialecosystemshime (accessed 11.12.15).

Pryde, S.E., Duncan, S.H., Hold, G.L., Stewart, C.S., Flint, H.J., 2002. The microbiology of butyrate formation in the human colon. FEMS Microbiol. Lett. 217, 133–9.

Qin, J., Li, R., Raes, J., Arumugam, M., Burgdorf, K.S., Manichanh, C., Nielsen, T., Pons, N., Levenez, F., Yamada, T., Mende, D.R., Li, J., Xu, J., Li, S., Li, D., Cao, J., Wang, B., Liang, H., Zheng, H., Xie, Y., Tap, J., Lepage, P., Bertalan, M., Batto, J.-M., Hansen, T., Le Paslier, D., Linneberg, A., Nielsen, H.B., Pelletier, E., Renault, P., Sicheritz-Ponten, T., Turner, K., Zhu, H., Yu, C., Li, S., Jian, M., Zhou, Y., Li, Y., Zhang, X., Li, S., Qin, N., Yang, H., Wang, J., Brunak, S., Doré, J., Guarner, F., Kristiansen, K., Pedersen, O., Parkhill, J., Weissenbach, J., Antolin, M., Artiguenave, F., Blottiere, H., Borruel, N., Bruls, T., Casellas, F., Chervaux, C., Cultrone, A., Delorme, C., Denariaz, G., Dervyn, R., Forte, M., Friss, C., van de Guchte, M., Guedon, E., Haimet, F., Jamet, A., Juste, C., Kaci, G., Kleerebezem, M., Knol, J., Kristensen, M., Layec, S., Le Roux, K., Leclerc, M., Maguin, E., Melo Minardi, R., Oozeer, R., Rescigno, M., Sanchez, N., Tims, S., Torrejon, T., Varela, E., de Vos, W., Winogradsky, Y., Zoetendal, E., Bork, P., Ehrlich, S.D., Wang, J., 2010. A human gut microbial gene catalogue established by metagenomic sequencing. Nature 464, 59–65.

Roberfroid, M., Gibson, G.R., Hoyles, L., McCartney, A.L., Rastall, R., Rowland, I., Wolvers, D., Watzl, B., Szajewska, H., Stahl, B., Guarner, F., Respondek, F., Whelan, K., Coxam, V., Davicco, M.-J., Léotoing, L., Wittrant, Y., Delzenne, N.M., Cani, P.D., Neyrinck, A.M., Meheust, A., 2010. Prebiotic effects: metabolic and health benefits. Br. J. Nutr. 104, 1–63.

Satokari, R.M., Vaughan, E.E., Akkermans, A.D., Saarela, M., de Vos, W.M., 2001. Polymerase chain reaction and denaturing gradient gel electrophoresis monitoring of fecal bifidobacterium populations in a prebiotic and probiotic feeding trial. Syst.Appl.Microbiol.

Sela, D. a, Chapman, J., Adeuya, A., Kim, J.H., Chen, F., Whitehead, T.R., Lapidus, A., Rokhsar, D.S., Lebrilla, C.B., German, J.B., Price, N.P., Richardson, P.M., Mills, D. a, 2008. The genome sequence of Bifidobacterium longum subsp. infantis reveals adaptations for milk utilization within the infant microbiome. Proc. Natl. Acad. Sci.

Sela, D.A., Chapman, J., Adeuya, A., Kim, J.H., Chen, F., Whitehead, T.R., Lapidus, A., Rokhsar, D.S., Lebrilla, C.B., German, J.B., Price, N.P., Richardson, P.M., Mills, D.A., 2008. The genome sequence of Bifidobacterium longum subsp. infantis reveals adaptations for milk utilization within the infant microbiome. Proc. Natl. Acad. Sci. U. S. A. 105, 18964–9.

Sierra, C., Bernal, M.-J., Blasco, J., Martínez, R., Dalmau, J., Ortuño, I., Espín, B., Vasallo, M.-I., Gil, D., Vidal, M.-L., Infante, D., Leis, R., Maldonado, J., Moreno, J.-M., Román, E., 2015. Prebiotic effect during the first year of life in healthy infants fed formula containing GOS as the only prebiotic: a multicentre, randomised, double-blind and placebo-controlled trial. Eur. J. Nutr.

Sivieri, K., Morales, M.L.V., Saad, S.M.I., Adorno, M.A.T., Sakamoto, I.K., Rossi, E.A., 2014. Prebiotic effect of fructooligosaccharide in the simulator of the human intestinal microbial ecosystem (SHIME® model). J. Med. Food.

Śliżewska, K., Kapuśniak, J., Barczyńska, R., Jochym, K., 2012. Resistant Dextrins as Prebiotic. Carbohydrates.

Sommer, F., Bäckhed, F., 2013. The gut microbiota — masters of host development and physiology. Nat. Rev. Microbiol.

Transparency Market Research, 2014. Prebiotics Market - Global Industry Analysis, Size, Share, Growth, Trends and Forecast, 2013 - 2019 [WWW Document]. URL http://www.transparencymarketresearch.com/prebiotics.html (accessed 12.2.15).

Turroni, F., van Sinderen, D., Ventura, M., 2011. Genomics and ecological overview of the genus Bifidobacterium. Int. J. Food Microbiol. 149, 37–44.

Underwood, M.A., German, J.B., Lebrilla, C.B., Mills, D.A., 2015. Bifidobacterium longum subspecies infantis: champion colonizer of the infant gut. Pediatr. Res. 77, 229–35.

Van den Abbeele, P., Belzer, C., Goossens, M., Kleerebezem, M., De Vos, W.M., Thas, O., De Weirdt, R., Kerckhof, F.-M., Van de Wiele, T., 2013. Butyrate-producing Clostridium cluster XIVa species specifically colonize mucins in an in vitro gut model. ISME J. 7, 949–61.

Van den Abbeele, P., Grootaert, C., Marzorati, M., Possemiers, S., Verstraete, W., Gérard, P., Rabot, S., Bruneau, A., El Aidy, S., Derrien, M., Zoetendal, E., Kleerebezem, M., Smidt, H., Van de Wiele, T., 2010. Microbial community development in a dynamic gut model is reproducible, colon region specific, and selective for Bacteroidetes and Clostridium cluster IX. Appl. Environ. Microbiol. 76, 5237–46.

Van den Abbeele, P., Van de Wiele, T., Verstraete, W., Possemiers, S., 2011. The host selects mucosal and luminal associations of coevolved gut microorganisms: A novel concept. FEMS Microbiol. Rev. 35, 681–704.

Venema, K., do Carmo, A.P., 2015. Probiotics and Prebiotics - Current Research and Future Trends, in: Venema, K., do Carmo, A.P. (Eds.), Probiotics and Prebiotics Current Research and Future Trends. Caister Academic Press, Norfolk, UK, pp. 3–12.

Ventura, M., Turroni, F., van Sinderen, D., 2015. Bifidobacteria of the Human Gut: Our Special Friends, in: Tuohy, K., Del Rio, D. (Eds.), Diet-Microbe Interactions in the Gut - Effects on Human Health and Disease. Academic Press, pp. 41–51.

Verhoeckx, K., Cotter, P., Kleiveland, C., Lea, T., Mackie, A., Requena, T., 2015. In Vitro Fermentation Models: General Introduction, in: Verhoeckx, K. (Ed.), The Impact of Food Bioactives on Health: In Vitro and Ex Vivo Models. Springer International Publishing, Cham, pp. 275–279.

Vilchez-Vargas, R., Geffers, R., Suárez-Diez, M., Conte, I., Waliczek, A., Kaser, V.S., Kralova, M., Junca, H., Pieper, D.H., 2013. Analysis of the microbial gene landscape and transcriptome for aromatic pollutants and alkane degradation using a novel internally calibrated microarray system. Environ. Microbiol. 15, 1016–39.

Vital, M., Penton, C.R., Wang, Q., Young, V.B., Antonopoulos, D. a, Sogin, M.L., Morrison, H.G., Raffals, L., Chang, E.B., Huffnagle, G.B., Schmidt, T.M., Cole, J.R., Tiedje, J.M., 2013. A gene-targeted approach to investigate the intestinal butyrate-producing bacterial community. Microbiome 1, 1–14.

Ward, R.E., Ninonuevo, M., Mills, D.A., Lebrilla, C.B., German, J.B., 2006. In Vitro Fermentation of Breast Milk Oligosaccharides by Bifidobacterium infantis and Lactobacillus gasseri. Appl. Environ. Microbiol. 72, 4497–4499.

Wiele, T. Van De, Abbeele, P. Van Den, Ossieur, W., Possemiers, S., Marzorati, M., 2015. The Simulator of the Human Intestinal Microbial Ecosystem (SHIME ®), in: Verhoeckx, K. (Ed.), The Impact of Food Bioactives on Health: In Vitro and Ex Vivo Models. Springer International Publishing, Cham, pp. 305–17.

Zhou, L., Foster, J.A., 2015. Psychobiotics and the gut-brain axis: in the pursuit of happiness. Neuropsychiatr. Dis. Treat. 11, 715–23.

Ziegler, E., Vanderhoof, J. a, Petschow, B., Mitmesser, S.H., Stolz, S.I., Harris, C.L., Berseth, C.L., 2007. Term infants fed formula supplemented with selected blends of prebiotics grow normally and have soft stools similar to those reported for breast-fed infants. J. Pediatr. Gastroenterol. Nutr. 44, 359–64.

VIII. APPENDIX

I

Table 18 – Conditions and donors associated to each bottle.

Condition / Bottle	Treatment		Donor fresh faecal inoculum									
	0 g/L	5 g/L	1	2	3	4	5	6	7	8	9	10
1 (CTRL)	X		X									
2 (TRT A)		X	X									
3 (TRT B)		X	X									
4	X			X								
5		X		X								
6		X		X								
7	X				X							
8		X			X							
9		X			X							
10	X					X						
11		X				X						
12		X				X						
13	X						X					
14		X					X					
15		X					X					
16	X							X				
17		X						X				
18		X						X				
19	X								X			
20		X							X			
21		X							X			
22	X									X		
23		X								X		
24		X								X		
25	X										X	
26		X									X	
27		X									X	
28	X											X
29		X										X
30		X										X

II

Table 19 – SHIME nutritional medium (g/L) for an optimal simulation of the nutrient intake in babies.

Nutritional medium components	**Stabilization and control**	**Treatment**				
		100/0	**90/10**	**75/25**	**50/50**	**0/100**
Pectin	1	1	1	1	1	1
Mucin	4	4	4	4	4	4
Starch	1	1	1	1	1	1
Glucose	1	1	1	1	1	1
Cellobiose	1	1	1	1	1	1
Proteose peptone	2	2	2	2	2	2
Pre-digested and pre-absorbed infant formula:						
• Lactose	2.1	2.1	2.1	2.1	2.1	2.1
• Casein	0.2	0.2	0.2	0.2	0.2	0.2
• Whey proteins (lactalbumin)	2.7	2.7	2.7	2.7	2.7	2.7
Treatment:						
* NMO	0.1		2.88	2.4	1.6	3.2
* GS	0.1	3.2	0.32	0.8	1.6	
TOTAL (g/L)	**15.2**	**18.2**	**18.2**	**18.2**	**18.2**	**18.2**